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Neonatal Vaccination: Role for Innate Immune Cell Interactions in BCG Vaccination

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Declaration

I declare that this thesis entitled 'Neonatal Vaccination: Role for Innate Immune Cell Interactions in BCG Vaccination' has been composed entirely by the candidate, Carly Hamilton. This work has been generated solely by myself as the result of my own original research and has not been submitted for any other degree or professional qualification. All sources of information have been acknowledged.

Carly Hamilton

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Bovine tuberculosis (TB), caused by *Mycobacterium bovis*, is increasing in incidence in the United Kingdom and detailed knowledge of host-pathogen interactions in the natural host is essential to facilitate disease control. Vaccination of neonatal calves with Bacille Calmette Guerin (BCG) induces a significant level of protection from infection with *M. bovis*. Since neonatal vaccination of humans with BCG induces activation of natural killer (NK) cells, and neonatal calves have high circulating numbers of these cells, it is proposed that NK cells are important in the response to BCG. Furthermore, NK cells play an important role in shaping adaptive immune responses through interactions with dendritic cells (DCs). The overall hypothesis of this project was that the enhanced efficacy of BCG in neonates is due to the increased number of NK cells, which through interactions with DCs can polarise Th1-type CD4+ and CD8+ T cell responses, both of which are involved in protection against *M. bovis* infection. Initially, the frequency and phenotype of NK cells across the blood, afferent lymph and the lymph nodes in steady-state conditions were compared. CD2- NK cells were the principal subset of NK cells migrating from the skin to the draining lymph node and were highly activated in afferent lymph and lymph nodes, compared with peripheral blood. It was also demonstrated that CD2- NK cells were the main subset of NK cells egressing from the lymph node via the efferent lymphatic vessel to return to circulation. Since many vaccines including BCG are delivered subcutaneously, NK cell responses in the blood and the skin draining afferent lymphatic vessel, lymph nodes and efferent lymphatic vessel were determined after BCG vaccination. Alterations in the frequency and receptor repertoire were evident following vaccination, supporting a role for NK cells during BCG vaccination of neonatal calves. To investigate the interactions of NK cells and BCG-infected DCs, *in vitro* co-cultures were established. CD2- NK cells were preferentially activated following culture with BCG-infected DCs and secreted high levels of IFN-γ. Overall, this thesis provides novel evidence that NK cells may re-circulate in steady-state conditions, play a role in BCG vaccination of neonatal calves, and that through interactions with BCG-infected DCs, may be involved in driving protective Th1-type adaptive immune responses.

Bovine tuberculosis (TB), caused by infection of cattle with *Mycobacterium bovis*, is increasing in incidence within the United Kingdom, with no vaccine currently licensed for use. Bacille Calmette Guerin (BCG) is the only vaccine licensed for use in humans against TB and is particularly effective when administered to infants. Similarly BCG vaccination of neonatal calves provides protection against infection. Young calves have an increased number of natural killer (NK) cells (white blood cells) which provide a rapid immune response to infection. In order to design better vaccines against TB, the immune response following vaccination must be understood. The hypothesis of this PhD project was that the enhanced efficacy of BCG in young animals is due to the increased number of NK cells which can contribute to protective immunity through interactions with dendritic cells (DCs). DCs are immune cells which are essential for initiation of immune responses. This project has demonstrated that NK cell frequency and receptor repertoire was altered following BCG vaccination of neonatal calves, supporting a possible role for NK cells during vaccination. Furthermore, this project also illustrated that a subset of NK cells can migrate from the tissues to the draining lymph nodes and this subset was also shown to preferentially interact with DCs infected with BCG. Therefore, NK cells may represent an effective target to induce protective immunity during BCG vaccination of neonatal calves.

Publications

Waters, R., Hope, J., **Hamilton, C**., Palmer, M., McNair, J., Skuce, R., Allen, A., Buddle, B., Villarreal-Ramos, B & Vordermeier, M. (2015). Immunopathogenesis of Mycobacterium bovis Infection of Cattle. Many Host of Mycobacteria: Tuberculosis, Leprosy, and other Mycobacterial Diseases of Man and Animals. Publisher: CABI.

Conference Presentations (Oral)

Hope J., Siddiqui N., Hammond J., Entrican G., & **Hamilton C**. (2015). Innate Immune Responses and Bovine Tuberculosis. Keystone Symposia, Immunity to Veterinary Pathogens: Informing Vaccine Development, Colorado, USA.

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****Presenting authors are underlined***

Ad85A	Adenovirus type 5 expressing *M. tb* antigen, Ag85A
AL	Afferent Lymph
ALDC	Afferent lymph dendritic cell
APC	Antigen presenting cell
APHA	Animal and Plant Health Agency
BCG	Bacille Calmette Guerin
BFA	Brefeldin A
BRSV	Bovine Respiratory Syncytial Virus
BSA	Bovine Serum Albumin
bTB	Bovine tuberculosis
BU	Biological unit
bp	Base pairs
CD	Cluster of differentiation
CFP-10	Culture filtrate protein - 10
CFU	Colony forming unit
CPDA	Citrate Phosphate Dextrose Adenine
Ct	Cycle threshold
DCs	Dendritic cells
DC – SIGN	Dendritic cell – specific ICAM-3 grabbing non integrin
DIVA	Differentiating infected from vaccinated animals
DMSO	Dimethyl sulfoxide
DTH	Delayed type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
EL	Efferent lymph
ESAT – 6	Early secreted antigenic target 6

ELISA	Enzyme - linked immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FSC	Forward scatter
GM-CSF	Granulocyte-macrophage colony stimulating factor
HEV	High endothelial venules
HIV	Human immunodeficiency virus
HSCs	Hematopoietic stem cells
IC	Isotype control
iDC	Immature dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IGRA	IFN-γ release assay
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ITIM	Immunoreceptor tyrosine-based inhibition motifs
KIR	Killer cell immunoglobulin-like receptor
KLRA	Killer cell lectin-like receptor subfamily A
KLRC	Killer cell lectin like receptor
LN	Lymph node
LRC	Leukocyte receptor complex
mAb	Monoclonal antibody
M. bovis	*Mycobacterium bovis*
MDR	Multi-drug resistant
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex

MIC A	MHC class I-related chain A
MIC B	MHC class I-related chain B
MIIC	MHC-II compartment
MNC	Mononuclear cells
MOI	Multiplicity of infection
MVA85A	Modified vaccinia virus Ankara expressing Ag85A
MR	Mannose receptor
M. tb	*Mycobacterium tuberculosis*
MVA	Modified vaccinia virus Ankara
NCR	Natural cytotoxicity receptor
NK	Natural Killer
NKC	Natural Killer Complex
NMS	Normal mouse serum
NTM	Nontuberculous mycobacteria
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PIM	Phosphatidylinositol mannoside molecules
PMA	Phorbol-myristate-acetate
PPD	Purified protein derivative
PRR	Pathogen recognition receptor
PSLN	Pre-scapular lymph node
RBCT	Randomised Badger Culling Trial
RD1	Region of difference 1
rhuAdV5	recombinant human replication-defective human adenovirus 5

ROI	Reactive oxygen intermediates
RT	Room temperature
S1P	Sphingosine-phosphate receptor
SRCR	Scavenger receptor cysteine-rich
SICCT	Single intradermal comparative cervical tuberculin
SSC	Side scatter
TB	Tuberculosis
TCM	Tissue culture medium
TCR	T cell receptor
TDR	Totally drug resistant
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UK	United Kingdom
ULBP	Unique long 16-binding proteins
V	Volts
WC	Workshop cluster
WHO	World Health Organisation
XDR	Extensively drug resistant

The introduction of this thesis covers a broad range of aspects of mycobacterial infection and immunity with specific aspects further expanded in each of the results chapters.

1.1 Human tuberculosis

Mycobacterium tuberculosis (*M. tb*), a Gram-positive, acid fast, aerobic bacillus is the aetiological agent of human tuberculosis (TB), a chronic bacterial infection of the respiratory tract. *M. tb*, alongside *M. bovis*, *M. bovis* BCG, *M. canettii*, *M. africanum*, *M. pinnipedii*, *M. microti*, *M. caprae* and the more recently defined *M. orygis* (van Ingen et al., 2012) collectively form the *M. tb* complex (Wirth et al., 2008). The species and sub-species within this genetically related complex cause TB in a number of mammalian hosts, with certain members of the *M. tb* complex exhibiting a more restricted host range than others. For example, human TB is most commonly caused by infection with *M. tb*, however *M. bovis* can infect a wide range of mammalian host species (Phillips et al., 2003). In 2006, the World Health Organisation (WHO) developed a Stop TB strategy, which aims to dramatically reduce the global burden of human TB by 2015 and to eliminate TB by 2050 (http://www.who.int/tb/strategy/en/ last accessed 09/09/15). Despite this, TB remains the leading cause of mortality due to a bacterial agent worldwide. In 2013, 9 million people developed TB and 1.5 million people died from this disease (WHO, 2014). A recognised risk factor for human TB is infection with human immunodeficiency virus (HIV) and of the 1.5 million people who died from TB in 2013, 360,000 were HIV positive. Additional risk factors for active TB include: diabetes, kidney disease, alcohol or drug abuse and other forms of immunosuppression. Of interest, it is estimated than one third of the world's population is latently infected with TB, which is a subclinical phase where the pathogen remains dormant (Dye et al., 1999). Of those with latent TB, only 5 to 10% will go on to develop active disease, which is usually a consequence of immunosuppression (Comstock et al., 1974, Vynnycky and Fine, 2000). Active TB disease can be broadly divided into three forms: pulmonary TB, extra-pulmonary TB and miliary TB. Individuals with pulmonary TB, defined by the presence of bacilli within the respiratory tract and lungs, present with a persistent cough, breathlessess,

fatigue, fever and weight loss. In severe cases, bacilli can spread to local lymph nodes, which is defined as extra-pulmonary TB or can be more widespread, defined as miliary TB (Harisinghani et al., 2000) .

1.1.1 Diagnostics for human TB

Human TB is diagnosed using a combination of acid-fast (Ziehl-Neelson) staining of sputum samples and chest X-ray screening in developing countries (Parsons et al., 2011). In developed countries, TB is identified using a tuberculin skin test (also called the Mantoux tuberculin skin test) whereby purified protein derivative (PPD) from *M. tb* is injected into the skin and the delayed type hypersensitivity response (DTH) is assessed 48 to 72 hours later. Secondly, IFN-γ release assays (IGRAs) such as the QuantiFERON®–TB Gold In-Tube test and T-SPOT®.TB test can be used as an alternative to the tuberculin skin test. The QuantiFERON®–TB Gold In-Tube test and the T-SPOT®.TB test measure the production of antigen-specific IFN-γ to *M.tb*-specific antigens using a whole blood assay and Enzyme-linked ImmunoSpot (ELISPOT) assay respectively. Sensitivity and specificity of these IGRAs ranged from 73-83% and 49-58% respectively (WHO, 2011).

1.1.2 Treatment for human TB

Active TB disease is treated with a combination of isoniazid, rifampicin, pyrazinamide and ethambutol for a minimum of 6 months or 12 months for pulmonary TB and extra-pulmonary TB respectively (www.nhs.uk last accessed 09/09/15). However, the emergence of multidrug-resistant strains which are resistant to isoniazid and rifampicin has resulted in the development of multidrug-resistant TB (MDR-TB) and in 2013, 480,000 people were diagnosed with MDR-TB (http://www.who.int/tb/challenges/mdr/mdr_tb_factsheet.pdf last accessed 09/09/15). Furthermore, the prevalence of cases of extensively drug-resistant TB (XDR-TB) and totally drug-resistant TB (TDR-TB) are increasing. The existence of these resistant strains further complicates the treatment of TB infection in humans and threatens the success of campaigns such as STOP-TB.

1.2 Bovine TB

Infection of cattle with *M. bovis* causes bovine TB (bTB) which is a major animal health problem. In the 1930s, approximately 15 to 20% of cattle in the United Kingdom (UK) were infected with *M. bovis*. The introduction of a nationwide bTB eradication programme in the 1960s resulted in a significant reduction in the prevalence of TB in cattle with 0.49% of all herds having a reactor in 1979 (Krebs JR, 1997). Nevertheless, despite continued routine testing, the incidence of bTB has increased in the UK since the early 1980s, particularly in regions of South West of England and South Wales (Figure 1.1). In 2010, 25,000 cattle were slaughtered at a cost of £91 million. It is estimated that bTB will cost the UK economy up to £1 billion over the next 10 years if effective control strategies are not implemented. Furthermore, bTB is a zoonotic disease and therefore poses a threat to human health (Cosivi et al., 1998). Prior to the introduction of milk pasteurisation in the 1930s, the majority of TB cases in humans were due to infection with *M. bovis* through consumption of infected milk (de la Rua-Domenech, 2006). However, a recent study assessed the global occurrence of zoonotic TB caused by *M. bovis* and concluded that the incidence of human TB as a result of zoonotic *M. bovis* infection was low (Muller et al., 2013).

Reasons for the increasing incidence of bTB in the UK are not clear, however it is likely to be due to a combination of problems with the available diagnostic tests (section 1.2.1) and wildlife reservoirs of infection. Wildlife reservoirs of infection pose significant risks for transmission of *M. bovis* infection to cattle. These include Eurasian badgers (*Meles meles*) in the UK and Ireland, brushtail possums (*Trichosurus vulpecula*) in New Zealand and white-tailed deer (*Odocoileus virginianus*) in the United States (Robinson et al., 2012). In 1997, an independent scientific commission set up by the UK government and chaired by Sir John Krebs, concluded that badgers were a significant source of *M. bovis* infection in cattle (Krebs JR, 1997). Furthermore, since the introduction of the badger protection act, the number of badgers has increased (http://www.legislation.gov.uk/ukpga/1992/51/contents last accessed 09/09/15). During the Randomised Badger Culling Trial (RBCT), which took place in England between 1998 and 2005, proactive culling of badgers reduced the incidence of bTB in cattle herds located within those areas where badgers were culled (Donnelly et al., 2007). However, in areas adjacent to the culling zones there was an increase in the

number of herd breakdowns, thought to be attributed to the dissemination of infected badgers, defined as the 'perturbation effect' (Wilson et al., 2011). Even if culling was deemed to be effective, it is not a feasible single solution for bTB control due to economical and ethical reasons. Other reasons which may explain the spread of bTB include housing of infected animals in confined spaces, movement of infected animals resulting in transmission of bTB across different regions and international trading of infected livestock (Waters et al., 2014).

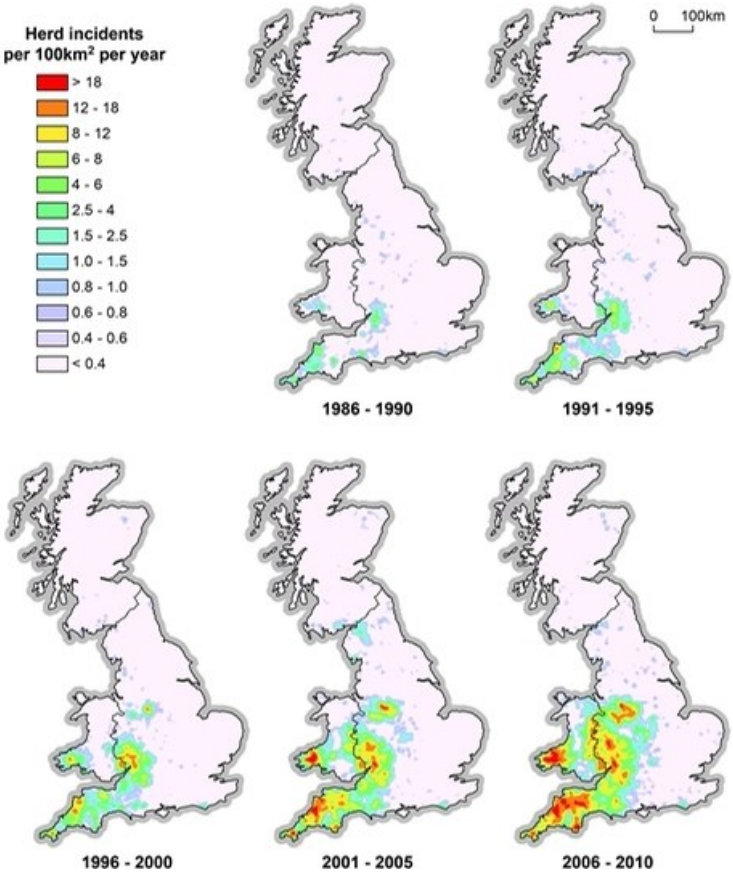


Figure 1.1 Bovine TB has increased in incidence across England and Wales from 1986 to 2010.

The incidence of bovine TB has increased in incidence since the mid-1980s to 2010 particularly in areas of South West of England and Wales. Data courtesy of Animal Plant and Health Agency (APHA). Copyright: University of Exeter.

1.2.1 Current bovine TB control measures

The principal test to diagnose bTB is the single intradermal comparative cervical tuberculin (SICCT) test, commonly known as the tuberculin skin test, which measures the cell-mediated immune response to purified protein derivatives from *M. avium* (PPD-a) and *M. bovis* (PPD-b) (Monaghan et al., 1994). Using the standard test interpretation, animals are considered to be infected with *M. bovis* if the reaction to PPD-b, administered subcutaneously, is 4mm greater than the reaction to PPD-a. If the standard test result is positive or inconclusive then the severe test interpretation is employed, whereby animals are deemed positive for bTB if the reaction to PPD-b is 2mm greater than the reaction to PPD-a. Animals which test positive in the SICCT (TB reactors) are slaughtered and the herd is placed under TB restrictions with repeated testing every 60 days until a clear herd test is achieved. In certain circumstances, an ancillary blood test can be employed alongside the skin test, for example when TB reactors have already been confirmed on the herd that is being tested. Samples of whole blood are stimulated with PPD-b for 24 hours and the production of antigen-specific IFN-γ present in the plasma supernatant is detected by ELISA (Wood and Jones, 2001). The SICCT is highly specific, with specificity estimated to be greater than 99.9%; however sensitivity is moderate, ranging from 50 to 60%. Sensitivity of the IFN-γ test (90%) is greater than the SICCT but the specificity of the IFN-γ test (96.7%) is insufficient for this to be the primary screening test for bTB. The IFN-γ test can detect infected animals that are negative to the SICCT.

Further complications involved in the diagnosis of bTB are that infection of cattle with *Mycobacterium avium* subspecies *paratuberculosis,* the causative agent of Johne's disease (Wadhwa et al., 2012), Bovine Viral Diarrhoea Virus or *Fasciola hepatica* conflicts the interpretation of the bTB diagnostic tests (Charleston et al., 2001, Flynn et al., 2007, Claridge et al., 2012). In addition, it is widely acknowledged that the IFN-γ test cannot be used in young animals due to non-specific production of IFN-γ in non-infected animals, caused by activation of NK cells (Olsen et al., 2005) or exposure to environmental, non-tuberculous mycobacteria (NTM) (Waters et al., 2006b).

To reduce the movement of infected cattle and subsequent spread of disease, movement restriction legislation is in place in Scotland, England and Wales

(https://www.gov.uk/government/publications/pre-movement-and-post-movement-tb-testing-of-cattle-in-great-britain last accessed 09/09/15). Pre-movement testing began in England and Wales in 2006 with pre-movement tests required 60 days before cattle movement and post-movement tests needing to be completed 60 to 120 days after movement of cattle. This should reduce the risk of further spread of bTB in England and Wales and allow Scotland to remain bTB free.

1.2.2 Treatment for bovine TB

bTB is rarely treated in domestic livestock and most *M. bovis*-infected animals will be slaughtered. Occasionally if animals belong to a rare or endangered species, or if they are considered to be zoological exhibits, then anti-mycobacterial therapy will be utilised.

1.3 Vaccination against TB

Vaccination is based on the induction of immunological memory and effective vaccines induce long-lived immune responses. The Krebs report highlighted the importance of developing a cattle vaccine and an associated diagnostic test (Krebs JR, 1997) which together, would greatly improve the control of bTB. However, despite extensive research into vaccination strategies, the development of novel vaccines to protect against TB in humans and cattle has proven extremely difficult (Hope and Vordermeier, 2005, Wilkie and McShane, 2015). Currently within the UK there are no licensed vaccines available for use in cattle and only one licensed vaccine for human TB.

1.3.1 Bacille Calmette Guerin (BCG) Vaccine

Bacille Calmette-Guerin (BCG), a live attenuated form of *M. bovis*, is the only TB vaccine available for use in humans. It is one of the world's most widely used vaccines and is administered to approximately 120 million children each year (Ritz and Curtis, 2009). Albert Calmette and Camille Guerin sub-cultured a virulent strain of bovine tubercle bacillus (*M. bovis*) on ox bile, glycerine and potato-soaked medium which resulted in attenuation of *M. bovis*. The potential use of BCG as a vaccine against *M. tb* infection in humans was demonstrated when Calmette and Guerin vaccinated 9 cows with BCG and showed that they were protected from challenge with *M. bovis*. The first

dose of BCG was administered to a newborn child in 1921. This original strain of BCG has been distributed to several laboratories worldwide and passaged under different conditions resulting in many daughter strains, including BCG Pasteur, BCG Tokyo and BCG Russia. These daughter strains differ in the deletions present and levels of attenuation which affect the virulence of each strain. For example region of difference 1 (RD1) is deleted in all BCG strains but BCG Pasteur is also missing RD2 and RD14 (reviewed in (Joung and Ryoo, 2013)). BCG confers variable protection against pulmonary TB, particularly when administered to adolescents or adults, with efficacy ranging from 0 to 80% (Fine, 1995, Colditz et al., 1995). Hypotheses which explain the variable efficacy of BCG in humans include: geographical location, exposure to environmental mycobacteria prior to vaccination which may stimulate an inappropriately biased immune response and distinct daughter strains of BCG that differ in their capabilities to stimulate protective immune responses (Andersen and Doherty, 2005). Routine BCG vaccination of adolescents stopped in the UK in 2005 and is currently only administered to babies from high risk families (http://www.bcgatlas.org/ last accessed 09/09/15).

In addition to the development of a cattle vaccine and an associated Differentiating Infected from Vaccinated Animals (DIVA) diagnostic test, the Krebs report also stated that a vaccine for badgers may improve control of bTB (Krebs JR, 1997). BCG was licensed for use in badgers in 2010 after 10 years of research into its efficacy and safety. It was subsequently demonstrated that BCG vaccination of free-living badgers reduced the incidence of positive serological tests by 73.8% (Chambers et al., 2011).

1.3.1.1. Neonatal vaccination of humans and cattle with BCG

Despite the variable efficacy observed following administration of BCG to adolescents or adults, BCG is particularly effective when delivered to infants and subsequently protects children against severe forms of childhood TB, such as TB meningitis and miliary TB (Trunz et al., 2006, Bonifachich et al., 2006). Similar to humans but unlike mice, cattle are born with a competent but immature immune system and therefore respond well to neonatal vaccination. Additionally, vaccination of neonatal calves overcomes the negative effects associated with exposure to environmental mycobacteria prior to vaccination. A number of studies have demonstrated that

experimental vaccination of neonatal calves with BCG imparts significant protection against challenge with *M. bovis* by reducing TB lesions in the lung and respiratory tract associated lymph nodes. Furthermore, neonatal vaccination with BCG reduces the number of *M. bovis* bacteria present in the tissues (Buddle et al., 1995a, Hope et al., 2005, Hope et al., 2011). A study investigating the duration of immunity induced by BCG vaccination of neonates showed that protection from *M. bovis* was evident 12 months post-vaccination, but not after 24 months (Thom et al., 2012). Revaccination of BCG-vaccinated calves at 24 months results in enhanced protection upon challenge with *M. bovis* (Parlane et al., 2014), providing evidence that homologous BCG revaccination is effective to boost waning immunity. Nevertheless, BCG sensitises cattle to the SICCT and therefore current legislation prevents BCG vaccination of cattle in the UK due to the inability of the available diagnostic tests to discriminate between infected and vaccinated cattle. Neonatal vaccination with BCG has also been shown to be partially protective against *M. bovis* infection in natural transmission settings during field studies in Ethiopia and Mexico with the duration of immunity lasting between 12 and 23 months (Ameni et al., 2010, Lopez-Valencia et al., 2010). Even though BCG has been shown to be effective to protect cattle against TB, similar to humans, the efficacy of BCG in cattle is variable across studies (reviewed in (Waters et al., 2012)). Possible reasons for the variable efficacy of BCG in cattle across a range of ages are firstly, BCG provides greater protection in *Bos indicus* breeds of cattle compared with *Bos taurus* (Ellwood and Waddington, 1972) therefore host genetic background may be important. For example, in well-controlled infection experiments where cattle are exposed to the same dose of *M. bovis*, there is a wide range of pathology scores observed likely reflecting host genetic differences. Secondly, different daughter strains of BCG may induce variable protection; however it has been shown that BCG strains Danish and Pasteur confer similar levels of protection (Hope et al., 2011, Wedlock et al., 2007). Finally, exposure to environmental mycobacteria prior to BCG vaccination may stimulate an inappropriately biased immune response.

1.3.1.2. DIVA tests

More sensitive and specific diagnostic tests, which can also differentiate infected from vaccinated animals (DIVA tests), are required to improve control of bTB. The specificity of the IFN-γ test can be improved via the inclusion of early secretory

antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) (Jones et al., 2010a) which are contained within RD1 that is deleted from all strains of BCG. A cocktail of ESAT-6, CFP-10 and Rv3615c (Rv3615c is not located on RD1 but secretion of Rv3165c is dependent on the Esx-1 secretion system which is contained within RD1 (Millington et al., 2011)) can be successfully used as a DIVA skin test (Whelan et al., 2010). Addition of a fourth antigen, Rv3020c further improved the sensitivity of the skin test (Jones et al., 2012). Rv3020c is present in both *M. bovis* and BCG genomes therefore the mechanisms underlying the improved sensitivity are unclear (Jones et al., 2010b). ESAT-6, CFP-10, Rv3615c and Rv3020c were identified as being effective DIVA test antigens by screening potential *M. bovis* specific antigens (Jones et al., 2010a, Jones et al., 2010b).

Alternatively, it was demonstrated that following stimulation of whole blood with PPD-b for 4 hours, *M. bovis*-infected cattle produced antigen-specific IFN-γ, whereas IFN-γ was not detected in the plasma supernatant of BCG-vaccinated cattle until 24 hours after stimulation (Sopp et al., 2008). This provides a simple and rapid method to distinguish *M. bovis*-infected from BCG-vaccinated cattle. Furthermore, active TB in humans can also be detected by this method (Sopp et al., 2008). Similarly, *M. bovis*-infected cattle produce antigen-specific IL-2 after stimulation with mycobacterial antigens (PPD-b, ESAT-6 and CFP-10) but BCG-vaccinated cattle do not, thus this test could also be applied to discriminate infected from vaccinated animals (DIVA tests) (Rhodes et al., 2013).

1.3.2 Heterologous prime-boost strategies in humans and cattle

Heterologous prime-boost vaccination strategies which involve priming the immune response with BCG and boosting with either DNA vaccines, proteins or live-attenuated viruses have been developed for both humans and cattle. Adenovirus type 5 expressing *M. tb* antigen, Ag85A (Ad85A) and modified vaccinia virus Ankara also expressing Ag85A (MVA85A) were shown to improve the efficacy of BCG alone, when utilised as the boosting agent in a heterologous prime-boost vaccination protocol, against *M. bovis* infection of cattle (Vordermeier et al., 2009). Boosting BCG primed animals with MVA85A (Vordermeier et al., 2004) and Ad85A (Vordermeier et al., 2006) results in the induction of strong cellular immunity by boosting IFN-γ responses to

Ag85A. MVA85A is one of the most clinically advanced human TB vaccination candidates and in 2009; MVA85A was the first subunit TB vaccine to enter into a phase IIb efficacy trial. Despite improved efficacy to BCG alone in four preclinical models (mice, cattle, guinea pigs and non-human primates), MVA85A did not improve the protection over BCG alone against TB disease or *M. tb* infection in BCG-vaccinated South African infants (Tameris et al., 2013). Reasons for this may be: a high burden of TB in the study site, environmental influences, co-infection with other infectious agents or the vaccine may be more efficacious in adolescents or in adults rather than infants. In order to design better vaccines and diagnostic tests it is vital to understand the pathology and immunology of human and bovine TB.

1.4 Pathogenesis of human and bovine TB

Cattle are a natural host for *M. bovis* and the immunology and pathology of bTB is similar to human TB, therefore studies of *M. bovis* infection in cattle may impart knowledge of TB in humans (Waters et al., 2011). Bacilli contained within aerosols infect humans and cattle primarily via the respiratory route however bacilli can also invade the host via other routes such as mucous membranes or breaks in the skin. *M. bovis* is highly virulent in cattle with as low as a single organism sufficient to establish infection (Dean et al., 2005). Bacilli employ an array of immune evasion strategies to enable intracellular survival and persistence. For example: (1) prevention of phagosome maturation and subsequent fusion with lysosomes to form phagolysosomes (2) inhibition of autophagy, apoptosis, IFN-γ receptor signalling and antigen processing and presentation to T cells (3) hindrance of escape from phagosome to cytoplasm and (4) inhibition of toxic oxygen and nitrogen intermediates (reviewed by (Ottenhoff, 2012)).

M. bovis bacilli persist within granulomas which are present as early as 7 to 15 days post-experimental infection with *M. bovis* and granuloma formation is employed by the host to limit proliferation of mycobacteria (Palmer et al., 2007). Granulomas are better studied in cattle due to the availability of tissues; however granulomas in infected cattle are similar to those present in infected humans. Granulomas are divided into Stage I, Stage II, Stage III and Stage IV and granulomas of all developmental stages can be present in the same section of tissue from 60 days post experimental

infection of cattle with *M. bovis*. Stage I granulomas are characterised by an accumulation of epithelioid macrophages with low numbers of Langhan's multinucleated giant cells, lymphocytes (mainly CD4+ T cells) and neutrophils. Stage II granulomas contain a central infiltrate of lymphocytes and neutrophils and unlike Stage I granulomas, have a thin fibrous capsule and a central area of necrosis. Stage III granulomas contain a thick fibrous capsule and a significant amount of central necrosis. Low numbers of CD8+ T cells, γδ T cells and B cells can be found in Stage I-III granulomas. Finally, Stage IV granulomas are characterised by the presence of multiple, merged granulomas with extensive necrosis and contain the highest number of bacilli (Palmer et al., 2007). Granulomas are most commonly found in the tissues and associated lymph nodes of the respiratory tract, head and thorax in naturally infected cattle (Neill et al., 1994).

1.5 Innate immune response to mycobacteria

The immune response to *M. tb* and *M. bovis* infection is not completely understood therefore greater understanding of the immune response to mycobacteria is essential to develop improved vaccines and diagnostic tests, to pinpoint correlates of protection and ultimately improve control of TB in both humans and cattle.

1.5.1 Macrophages

Macrophages are a diverse population of specialized phagocytic cells which are essential for host defense, homeostasis and wound repair. They are derived from bone marrow precursors and circulating blood monocytes, which differentiate into resident macrophages or dendritic cells (DCs) upon tissue entry (Verschoor et al., 2012). Macrophages are considered the major host cells for mycobacteria *in vivo* and due to the respiratory route of entry employed by mycobacteria, alveolar macrophages play a pivotal role during anti-mycobacterial immune responses (Pollock and Neill, 2002). Macrophages express an array of cell surface receptors that recognize mycobacteria including: Toll-like receptors (Means et al., 1999); the macrophage mannose receptor that recognizes mannosylated glycoproteins; Fc receptors binding opsonized cells and complement receptors (Ernst, 1998). Signaling pathways are activated as a result of mycobacterial recognition. Within bovine alveolar macrophages, up-regulation of genes associated with the transcription factor NFκB are evident early post-*M. bovis*

infection (Widdison et al., 2011). Macrophages ingest mycobacteria by phagocytosis and the bacilli are subsequently targeted to the phagosome where fusion with the lysosome occurs, resulting in bacterial destruction. However, mycobacteria can also evade the immune response and prevent phagolysosome fusion within macrophages. Nevertheless, activation of macrophages by IFN-γ from T cells and NK cells can partially block the arrest of phagolysosome fusion. Similarly, IFN-γ stimulates autophagy within *M. tb*-infected macrophages which also prevents arrest of phagolysosome fusion, thus inhibiting survival of bacteria (Gutierrez et al., 2004). Activated macrophages release reactive oxygen or nitrogen intermediates. For example, bovine alveolar macrophages stimulated with *M. bovis* express inducible nitric oxide synthase (iNOS) that catalyses the production of nitric oxide (NO) which aids macrophage killing of mycobacteria (Widdison et al., 2007). Inflammatory chemokines are also produced by *M. bovis*-infected macrophages which attract lymphocytes to the lungs and contribute to granuloma formation (Widdison et al., 2011).

1.5.2 Dendritic cells

DCs are a heterogeneous population of potent antigen-presenting cells (APC) which are essential mediators of immunity (Banchereau and Steinman, 1998) and tolerance (Steinman et al., 2003). Stem cell precursors in the bone marrow give rise to circulating myeloid or lymphoid precursors that enter tissues and reside as immature DCs. Monocytes, macrophages and DCs share a common progenitor (Fogg et al., 2006). Flt3L drives differentiation of DCs (Schmid et al., 2010) and loss of Flt3L (McKenna et al., 2000), Flt3 (Waskow et al., 2008) or downstream signalling molecule STAT3 (Laouar et al., 2003) reduces the number of DCs *in vivo*. DCs are predominantly found underlying body surfaces including the skin, intestine and the trachea. Immature DCs are specialised to capture antigen by a number of different routes including phagocytosis, macropinocytosis and endocytosis via clathrin-coated pits or caveolae (Werling et al., 1999). *M. tb* gains entry to human monocyte-derived DCs through binding to DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) (Tailleux et al., 2003). Likewise, binding of BCG- fluorescein isothiocyanate (FITC) to bovine DCs was reduced in the presence of a polyclonal antibody to DC-

SIGN revealing a role for DC-SIGN in uptake of BCG by bovine DCs (Yamakawa et al., 2008). TLR2, TLR4 and TLR9 have also been associated with the uptake and recognition of mycobacteria by DCs (Means et al., 1999, Tsuji et al., 2000, von Meyenn et al., 2006). DCs act as sentinels and respond to infection, inflammatory signals or tissue damage by migrating away from the periphery towards draining lymph nodes where they present antigen and initiate primary T cell mediated immune responses which is a unique feature of DCs (Steinman, 1991). *M.tb*-infected DCs migrate to the lung draining lymph nodes by 8 to 12 days post-infection (O'Garra et al., 2013). CCR7 expression by antigen-exposed DCs allows migration from the periphery to the draining lymph node via afferent lymphatic vessels, in response to the chemo attractants CCL19 and CCL21 (Rot and von Andrian, 2004). During migration, DCs undergo maturation which is characterised by reduced endocytosis and an augmented expression of major histocompatibility complex (MHC) molecules, costimulatory molecules and adhesion molecules. Following infection of human DCs with *M. tb*, expression of CD54, CD40, CD80 and MHC class I molecules were increased. In addition to increased expression of various cell surface molecules, DCs exposed to *M. tb* bacilli produce pro-inflammatory cytokines which induce T cell immune responses, such as TNF-α, IL-1 and IL-12 (Henderson et al., 1997). Likewise monocyte-derived DCs from cattle infected with *M. bovis* and BCG Pasteur up-regulated MHC class II, CD40 and CD80 in conjunction with increased production of TNF-α, IL-10 and IL-12, therefore suggesting increased capacity to stimulate antigen-specific T cells (Hope et al., 2004).

Bovine DCs comprise phenotypically distinct subsets which are of myeloid origin (Howard and Hope, 2000, Miyazawa et al., 2006) however a population of lymphoid derived plasmacytoid DCs have been identified in cattle (Reid et al., 2011). Bovine monocyte-derived DCs are generated *in vitro* from blood CD14+ monocytes which are cultured with recombinant bovine granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 to obtain an immature population (Werling et al., 1999). Alternatively, bone marrow-derived DCs can be obtained by culturing bone marrow progenitor cells with Flt-3L (Hope et al., 2000b). Afferent lymph derived DCs (ALDCs) can be studied in cattle via cannulation of pseudo-afferent lymphatic vessels (Hope et al., 2006, Emery et al., 1987) which allows collection of *ex vivo* DCs draining

sites of vaccination or infection. Sub-populations of ALDCs draining the skin (Howard et al., 1997, Brooke et al., 1998, McKeever et al., 1991) and mucosal surfaces have been described which have differential capacity not only to stimulate T cell responses (Howard et al., 1997) but which also display divergent capacities to interact with mycobacteria. Monocyte-derived DCs infected with BCG induced effective memory CD4+ and CD8+ T cell responses (Hope et al., 2000a). However, assessment of *ex vivo* populations of DCs isolated from afferent lymphatic vessels revealed that only a subset could uptake and present antigens from BCG (Hope et al., 2012). This could impact vaccine efficacy or the induction of protective immunity to infection.

1.5.3 Natural Killer (NK) Cells

NK cells are large granular lymphocytes which were identified in the 1970s by their ability to lyse malignant or transformed cells without prior sensitisation (Kiessling et al., 1975). This heterogeneous cell population has diverse roles in the immune system and are the first line of defence in the control of viruses, bacteria and parasites (Junqueira-Kipnis et al., 2003, Cerwenka and Lanier, 2001, Lieke et al., 2004, Artavanis-Tsakonas and Riley, 2002). NK cells are derived from self-renewing pluripotent hematopoietic stem cells (HSCs) that reside in the bone marrow. NK cells have two main effector functions, firstly cytotoxicity to target cells through the release of preformed granules containing perforin and granulysin. For example, it was shown that human NK cells kill *M. tb* in a contact-dependent manner by releasing granules containing perforin and granulysin (Lu et al., 2014). Secondly, NK cells are a significant source of immunoregulatory cytokines, primarily IFN-γ but also TNF-α, GM-CSF (Fehniger et al., 1999, Cooper et al., 2001b, Boysen et al., 2006), IL-10 (Fehniger et al., 1999, Cooper et al., 2001b) and IL-22 (Cella et al., 2009, Dhiman et al., 2009).

An increasing body of evidence demonstrates that NK cells are not simply cells confined to the innate arm of the immune response; rather they bridge the innate and adaptive responses. In line with this, NK cells have a number of similar developmental features with cells of the adaptive immune system. NK cells, T cells and B cells are derived from the common lymphoid progenitor (Kondo et al., 1997) and all require IL-2, IL-7 and IL-15 for their development, homeostasis and survival (Di Santo, 2006).

In addition, NK cells undergo education in the bone marrow which is very similar to the process of T cell development in the thymus (Sun and Lanier, 2011, Orr and Lanier, 2010). Furthermore, NK cells have been shown to have functional features of adaptive immunity including immunological memory (Sun et al., 2009, O'Leary et al., 2006, Cooper et al., 2009, Paust and von Andrian, 2011). The first evidence of NK cell memory was demonstrated in 2006 when mice devoid of T and B cells acquired antigen-specific immunological memory to hapten-based contact sensitizers. This was found to be mediated by a subset of NK cells localised in the liver (O'Leary et al., 2006). NK cells respond to CD4+ T cell derived IL-2 after rabies vaccination of humans (Horowitz et al., 2010) or following infection of mice with *Leishmania major* (Bihl et al., 2010), further strengthening the role of NK cells in adaptive immune responses. Furthermore, discrete subsets of human NK cells have also been shown to express MHC class II after stimulation with IL-2, thus NK cells may present antigen to CD4+ T cells (Evans et al., 2011). Lastly, NK cells can bridge the innate and adaptive immune response through interactions with populations of accessory cells such as DCs. These interactions are reciprocal in nature and can polarise the outcome of the adaptive immune response (Cooper et al., 2004).

1.5.3.1. Human NK cells

NK cells comprise 10-15% of peripheral blood lymphocytes in humans (Trinchieri, 1989). Frequencies of NK cells are highest within cord blood and then undergo a progressive decline during the first year of life. Human NK cells are defined by an absence of CD3 and can be broadly subdivided based on their differential expression of the cell surface markers CD56 and CD16 (FcγRIII) (Robertson and Ritz, 1990, Cooper et al., 2001a). Cells with a low expression of CD56 ($CD56^{dim}$) and high levels of CD16 comprise the majority (~90%) of human NK cells present within peripheral blood. The remaining ~10% of NK cells are classified as $CD56^{bright}$ with a low or negative expression of CD16. Due to their high expression of CD16 which mediates antibody-dependent cellular cytotoxicity, $CD56^{dim}$ NK cells are more cytotoxic than $CD56^{bright}$ NK cells (Nagler et al., 1989). By contrast $CD56^{bright}$ NK cells, which are the predominant NK cell subset found within lymph nodes, produce increased amounts of immunoregulatory cytokines including IFN-γ, compared with $CD56^{dim}$ NK cells

(Cooper et al., 2001b). Furthermore, $CD56^{bright}$ NK cells have a higher proliferative capacity in response to IL-2 (Baume et al., 1992).

1.5.3.2. Murine NK cells

CD56 is not expressed in mice therefore subsets of NK cells are distinguished by the expression of NK1.1 or CD49b (Arase et al., 2001) and can be further subdivided based on CD27 and CD11b expression (Chiossone et al., 2009). NK cells are present within the lymph nodes, liver and lungs of mice with the largest absolute number of NK cells found within the spleen (Gregoire et al., 2007). CD27+ NK cells are the principal subset of NK cells found within lymph nodes and in contrast, CD27- NK cells are the main subset in the blood and spleen (Inngjerdingen et al., 2011). Most laboratory mice have very few lymph node derived NK cells which is in contrast with feral mice who have a high frequency of primed NK cells within peripheral lymph nodes (Boysen et al., 2011).

NK cells are responsive during mycobacterial infection of humans and mice *in vivo* and important functional roles for NK cells in response to mycobacteria have been described (reviewed in (Esin and Batoni, 2015)). NK cells are recruited to the site of mycobacterial infection during pulmonary (Portevin et al., 2012) and extra-pulmonary TB in humans (Schierloh et al., 2009). Furthermore, a potential role for NK cell and γδ T cell derived IFN-γ during BCG immunisation of infants has been recently defined (Zufferey et al., 2013). Following infection of mice with *M. tb* or BCG, increased numbers of activated NK cells are recruited to the lungs where they secrete IFN-γ, however depletion of NK cells has no effect on the bacterial load within the lungs suggesting a level of redundancy (Junqueira-Kipnis et al., 2003). In BCG-immunised mice, NK cells control bacterial replication and enhance T cell responses through their secretion of IFN-γ and IL-22 (Dhiman et al., 2012). Taken together, this highlights the importance of innate effector cells such as NK cells, during anti-mycobacterial immune responses.

1.5.3.3. Bovine NK cells

NK cells are located within the spleen, lung, liver and lymph nodes of cattle (Storset et al., 2004, Boysen et al., 2008). Within bovine peripheral blood, NK cells represent

0.5%-10% of the total lymphocyte population with an increased prevalence in neonatal calves, particularly those aged between 8-120 days old (Kulberg et al., 2004, Graham et al., 2009). The high number of NK cells found in neonates is thought to be a mechanism whereby the innate immune response is able to compensate for the immature state of the adaptive immune system. Similar to mice, bovine NK cells do not transcribe CD56 (Endsley et al., 2006). NKp46 (NCR1; CD335), a natural cytotoxicity receptor expressed exclusively by NK cells, is recognised as a pan-species marker used to identify NK cells (Walzer et al., 2007c). The development of a monoclonal antibody (mAb) specific to this receptor has allowed NK cells to be studied in cattle (Storset et al., 2004). Prior to this, a population of NK-like CD8+ cells were described in cattle but these could not be definitively identified as NK cells until the mAb specific for NKp46 was developed (Hope et al., 2002b). Populations of bovine NK cells which lack NKp46 expression have also been detected, and are thought to exist in an immature state (Graham et al., 2009). Similar to humans, bovine NK cells lack expression of CD3 and can be subdivided into two subsets based on their differential expression of CD2, an adhesion molecule expressed by T cells and NK cells (Storset et al., 2004). As observed in mice (Nakamura et al., 1990) and humans (Chan et al., 1989), CD2 expression appears to be redundant for the function of bovine NK cells. The majority of peripheral blood derived NK cells are CD2+ and a small population have low or no expression of CD2 (referred to as CD2- herein). In contrast to peripheral blood, CD2- NK cells are the predominant subset found within lymph nodes and this subset has also been defined as the major NK cell subset present within skin draining afferent lymphatic vessels (Lund et al., 2013). CD2- NK cells have a higher expression of the activation markers CD25 and CD44, an increased proliferative capacity and enhanced ability to produce IFN-γ in comparison to their CD2+ counterparts. However, both subsets have equal cytotoxic capacities (Boysen et al., 2006). Parallels have been drawn between the CD2- subset of bovine NK cells and the $CD56^{bright}$ CD16- subset of human NK cells (illustrated in Figure 1.2).

There is considerable evidence in the literature that bovine NK cells play key roles in the immune response to mycobacteria. For example, bovine NK cells stimulated with IL-2, IL-15, IL-2/12 or IL-12/15 restrict the replication of BCG within monocyte-derived and alveolar macrophages (Endsley et al., 2006). As mentioned in section

1.5.3, NK cells are found at the interface between innate and adaptive immunity and accordingly, can interact with populations of accessory cells such as macrophages or DCs. Bovine NK cells proliferate and produce IFN-γ in response to interactions with *M. bovis*-infected macrophages, which enhances IL-12 and NO production by activated macrophages (Denis et al., 2007). Similarly, through interactions with BCG-infected splenic DCs, bovine NK cells acquire cytotoxic activity and produce IFN-γ (Bastos et al., 2008). More recently, reciprocal interactions between NK cells and DCs in the context of *M. bovis* were demonstrated, with preferential stimulation of the CD2- subset of NK cells (Siddiqui and Hope, 2012).

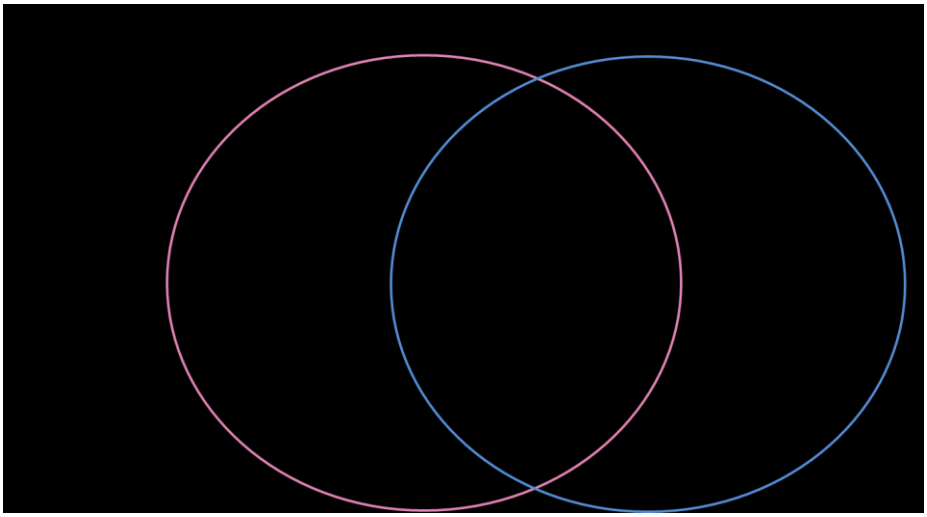


Figure 1.2 Similarities and differences between bovine CD2- NK cells and human CD56^{bright} NK cells

CD2- bovine NK cells and CD56^{bright} human NK cells are both localised in afferent lymph and lymph nodes. Furthermore, both subsets express CD25, CD44, CXCR4 and CCR7, proliferate after culture with IL-2 and produce IFN-γ. These subsets differ in their cytotoxic capabilities as CD2- NK cells are cytotoxic, however CD56^{bright} NK cells are weakly cytotoxic.

1.5.4 NK cell receptors

Unlike B and T cells, NK cells do not express receptors that require somatic gene rearrangements to generate receptor diversity (Lanier, 2005). Instead, NK cell function and maintenance of self-tolerance is determined by a complex interplay between a plethora of activating and inhibitory NK cell receptors (Long et al., 2013). These germ-line encoded receptors recognise MHC class I molecules, MHC class I-like molecules, stress-related molecules, costimulatory ligands and cytokines (Vivier and Ugolini, 2011, Ljunggren and Karre, 1990, Lanier, 1997). By using mass cytometry, it was demonstrated that host genetic background determines the expression of inhibitory receptors and the repertoire of activating receptors is influenced by the environment. This suggests that inhibitory receptors may be the key receptors involved in maintenance of self-tolerance and the response to pathogens may be controlled by activating receptors (Horowitz et al., 2013). NK cells can also eliminate target cells that fail to express self-MHC class I molecules during a phenomenon known as the 'missing self' hypothesis (Ljunggren and Karre, 1990) (Figure 1.3C). This paradigm has since been updated as it is now clear that in addition to the inhibitory signals, activating signals are also necessary for NK cell killing (Lanier, 2005). NK cell receptors contain extracellular domains which recognise MHC class I molecules and can be structurally divided into those containing either C-type lectin-like domains or Ig-like domains which are encoded within the NK complex (NKC) and leukocyte receptor complex (LRC) respectively. Both of these groups contain receptors capable of transmitting activating and inhibitory signals.

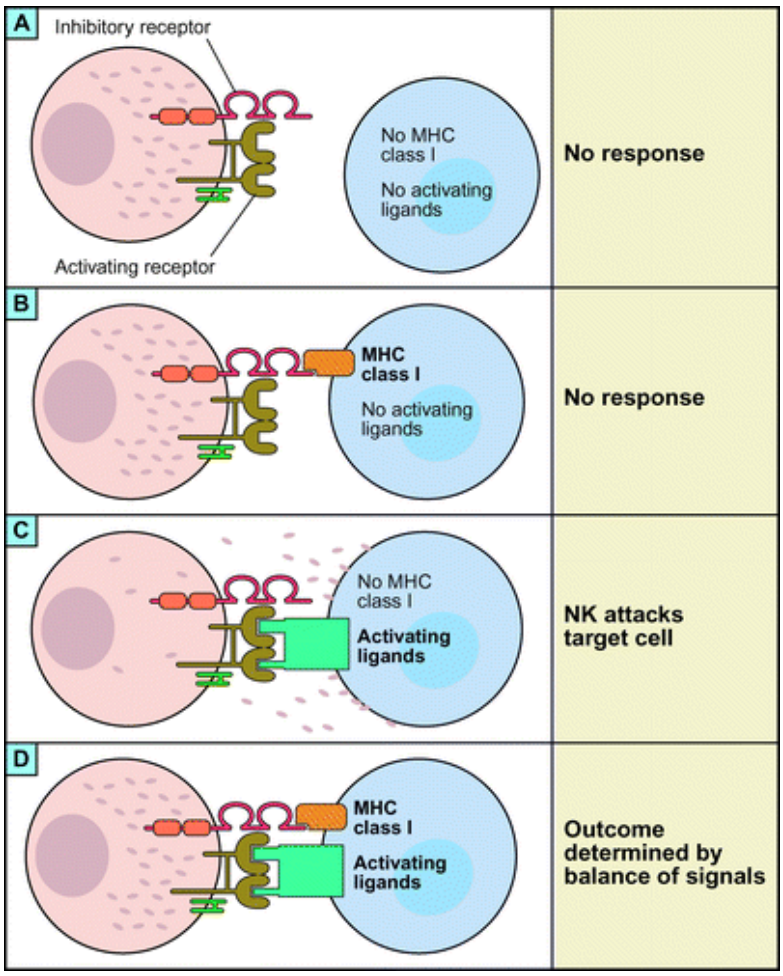


Figure 1.3 The balance between activating and inhibitory receptors expressed by NK cells

NK cells expressing activating and inhibitory receptors which do not detect activating ligands or MHC class I on a target cell remain unresponsive (A). If NK cell inhibitory receptors recognise MHC class I on a target cell, NK cells do not respond (B). However, if MHC class I is downregulated by a target cell then activating receptors are ligated and the NK cell attacks the target cell (C). Finally, if the target cell expresses ligands for both activating and inhibitory receptors, the outcome is determined by the balance between the strength of the two signals (D). Taken from (Lanier, 2005).

1.5.4.1. Inhibitory receptors

Inhibitory receptors which recognise MHC class I comprise the Killer Cell Immunoglobulin-Like Receptors (KIRs) in humans and primates, Ly49 (KLRA) receptors in rodents and CD94/NKG2 receptors. To date, cattle have the most expanded NK cell receptor gene family and are the only species to have significantly expanded the *KIR* and *CD94/NKG2* genes (Figure 1.4) (Guethlein et al., 2007, McQueen et al., 2002, Storset et al., 2003, Birch and Ellis, 2007, Dobromylskyj et al., 2009). Unlike non-primate mammals whereby the LRC contains one or no *KIR* genes (Hammond et al., 2009, Futas and Horin, 2013, Gagnier et al., 2003, Sambrook et al., 2006, Takahashi et al., 2004), cattle *KIR* have evolved independently with two distinct lineages denoted by L and X: *3DL* and *3DX* (Guethlein et al., 2007). The *3DL* lineage *KIR* gene is a single, conserved *KIR* gene and the second lineage consists of multiple *3DX* lineage *KIR* genes. This is in contrast with humans and higher primates which have expanded *3DL* lineage *KIR* genes but have a single, conserved *3DX* lineage *KIR* gene (Guethlein et al., 2007, Sanderson et al., 2014). KIR receptors, which belong to the Ig superfamily, can transmit activating signals through their short cytoplasmic tail which contains a charged amino acid residue that conveys activating signals upon binding to DAP12. However, KIR receptors primarily deliver inhibitory signals through the presence of two immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Thielens et al., 2012). Mice lack *KIR* genes but express C-type lectin Ly49 receptors which are functionally similar to the human KIR receptors (Lanier, 2005). To date, 18 *KIR* genes have been identified in cattle which are mainly inhibitory (Sanderson et al., 2014).

Ly49 (KLRA) receptors contain C-type lectin-like domains and are expressed on the surface of NK cells as disulphide-linked homodimers (Wong et al., 1991). They recognise MHC class I and MHC class I-like molecules and consist of both activating and inhibitory receptors. To date, no functional *Ly49* genes have been identified in humans, however 23 *Ly49* genes and 6 *Ly49* genes have been identified in mice and horses respectively (Berry et al., 2014, Takahashi et al., 2004). A single copy *Ly49* gene exists in cattle, which is highly polymorphic (McQueen et al., 2002, Dobromylskyj et al., 2009).

CD94-NKG2 receptor complexes are also C-type lectin-like receptors which consist of disulphide-linked heterodimers of CD94 and NKG2 molecules with CD94 required for surface expression of NKG2 family members (Lazetic et al., 1996). In humans, CD94 forms a heterodimer with NKG2A or NKG2C to transmit inhibitory or activating signals respectively. Both CD94/NKG2A and CD94/NKG2C bind to HLA-E (Braud et al., 1998). Similar to *KIR* lineage *3DX*, *NKG2* and *CD94* are significantly expanded in cattle and contain 2 *CD94* genes, 7 *NKG2A* genes and 2 *NKG2C* genes (Figure 1.3) (Birch and Ellis, 2007). It is has not been defined if CD94 forms heterodimers with NKG2A or NKG2C in cattle.

Species	Leukocyte receptor complex		Natural killer complex		
	KIR 3DL	*KIR 3DX*	*Ly49 (KLRA)*	*NKG2 (KLRC)*	*CD94 (KLRD)*
Cattle					
Pigs					
Horses					
Humans		Ψ	Ψ		
Higher primates					
Lemurs	Ψ				
Mice	Ψ				
Rats					
Cats	Ψ				
Dogs					
Seals					

Figure 1.4 Comparison between NK cell receptor genes in LRC and NKC across species

Blue = gene presence, pink = significant expansion, blank = gene is absent in the genome, ψ = pseudogene, orange = limited expansion. Taken from (Ellis and Hammond, 2014).

1.5.4.2. Activating receptors

The natural cytotoxicity receptors (NCRs) comprise a family of activating receptors belonging to the Ig superfamily. In the late 1990s it was discovered that human NK cells express three NCRs: NKp46 (NCR1; CD335) (Sivori et al., 1997), NKp44 (NCR2; CD336) (Vitale et al., 1998) and NKp30 (NCR3; CD337) (Pende et al., 1999). Resting human NK cells constitutively express NKp46 and NKp30, however NKp44 expression is induced following activation (Vitale et al., 1998). NKp44 is not present in mice or cattle (Cantoni et al., 1999, Hammond, 2012) and mice also lack expression of NKp30 (Hollyoake et al., 2005). NKp30 is the most diverse of all the NCRs and is involved in human NK cell lysis of immature DCs (Ferlazzo et al., 2002). Bovine NKp30 has been demonstrated to be expressed following cytokine stimulation (Hammond, 2012).

NK cells can be activated directly by pathogens such as mycobacteria through direct interactions with NCRs. For example, expression of NKp44 by human $CD56^{bright}$ NK cells allows BCG to bind directly to NK cells and induce proliferation, cytotoxicity and production of IFN-γ (Esin et al., 2008, Esin et al., 2004). Similarly, NKp44 expressed by human NK cells is able to interact directly with components of the *M. tb* cell wall (Esin et al., 2013). In contrast with NKp44 which can bind directly to *M. tb* cell wall components, NKp46 binds to vimentin expressed by *M. tb*-infected monocytes, resulting in lysis of *M. tb*-infected monocytes (Garg et al., 2006, Vankayalapati et al., 2002, Vankayalapati et al., 2005).

Another activating receptor is NKG2D which is expressed as a disulphide-linked homodimer on the surface of NK cells. Ligands of human NKG2D are the stress-induced molecules MHC class I-related chain A and B (MICA/MICB) and the unique long 16-binding proteins (ULBP) (Bottino et al., 2005). NKG2D expression by human NK cells permits lysis of *M. tb*-infected alveolar macrophages through recognition of ULPB (Vankayalapati et al., 2005).

1.5.5 NKT cells

NKT cells are a small subset of lymphocytes which have shared attributes of both NK cells and T cells. Classical T cells recognise MHC-presented peptides, however NKT

cells recognise lipids/glycolipids presented by CD1d molecules and a common NKT ligand is α-galactosylceramide (αGalCer). Following activation, NKT cells can produce significant amounts of Th1 (IFN-γ) and Th2 (IL-4 and IL-13) cytokines. NKT cells are well characterised in humans and mice, however their presence in cattle remains controversial. CD1d presents antigens to NKT cells in humans and mice but initially it was thought CD1d-restricted NKT cells did not exist in cattle due the presence of a non-functional *CD1d* gene (Van Rhijn et al., 2006). However, it has since been demonstrated that the bovine *CD1d* gene is expressed and translated *in vivo* (Nguyen et al., 2013). Furthermore, a population of bovine lymphocytes co-expressing NKp46 and CD3 were described, suggesting a population of NKT-like cells exists in bovine peripheral blood (Connelley et al., 2014). Recently, it was shown that bovine NKT-like cells can be activated by highly purified phosphatidylinositol mannoside (PIM) molecules isolated from *M. tb*, thus showing evidence for lipid-specific NKT activation for the first time in cattle (Pirson et al., 2015).

1.5.6 γδ T cells

γδ T cells, which have a T cell receptor (TCR) consisting of one γ and one δ chain, constitute a minor fraction of the lymphocyte population in humans and mice and represent approximately 10% of the circulating lymphocytes present. However, γδ T cells are a major subset of lymphocytes in ruminants, constituting approximately 60% of circulating T cells in young animals (Davis et al., 1996). In parallel with NK cells, numbers of γδ T cells are elevated in young calves and decline with age (Kulberg et al., 2004). Bovine γδ T cells are divided into distinct subsets based upon their expression of isoforms of Workshop Cluster 1 (WC1), a transmembrane glycoprotein and member of the scavenger receptor cysteine rich (SCRC) superfamily. The majority of blood derived γδ T cells express WC1 (Rogers et al., 2005) and lack expression of CD2 and CD8. A smaller proportion of circulating γδ T cells are WC1- CD8+ cells. Recently, it was proposed that bovine circulating γδ T cells are a major regulatory T cell population through their expression of IL-10 which supports the role of γδ T cells as key players in immune regulation in humans and mice (Guzman et al., 2014).

Considerable evidence exists to suggest that γδ T cells play a key role in the immune response to mycobacterial infection. Following BCG vaccination of calves, the

percentage of WC1+ γδ T cells present in the peripheral blood increased at 2 weeks post-vaccination alongside increased production of IFN-γ (Buza et al., 2009). Similarly, the percentage of IFN-γ producing WC1+ γδ T cells were elevated in the tissues of the lungs and upper respiratory tract following intranasal BCG vaccination (Price et al., 2010). In terms of virulent *M. bovis* infection, the frequency of peripheral blood derived WC1+ γδ T cells declines following *M. bovis* infection of cattle, thought to be attributed to their movement out of the circulation to the site of infection (Pollock et al., 1996). Furthermore, depletion of WC1+ γδ T cells from the circulation and respiratory tract of calves, prior to infection with *M. bovis*, had no effect on disease severity or granuloma formation. However, these studies revealed a role for WC1+ γδ T cells in the polarisation of a Th1 immune response during *M. bovis* infection (Kennedy et al., 2002).

In conjunction with NK cells, WC1+ γδ T cells also bridge innate and adaptive immune responses through interactions with APCs. During *in vitro* studies, WC1+ γδ T cells exhibited enhanced expression of CD25 and MHC class II in parallel with increased production of IFN-γ after co-culture with *M. bovis*-infected DCs. Levels of IL-12 secreted by *M. bovis*-infected DCs were also augmented after culture with WC1+ γδ T cells highlighting the reciprocal nature of the interaction between WC1+ γδ T cells and *M. bovis*-infected DCs (Price and Hope, 2009).

1.6 Adaptive immune response to mycobacteria

M. tb has the ability to delay onset of adaptive immune responses in mice or humans (Ottenhoff, 2012) and similarly, adaptive responses are delayed following *M. bovis* infection of cattle (Waters et al., 2009). The adaptive immune response is initiated when mature, mycobacteria-infected DCs arrive in the lung draining lymph nodes around day 11 post-infection where recognition of T cells can occur (Flynn, 2004).

1.6.1 αβ T cells

1.6.1.1. CD4+ T cells

T cells bearing the αβ TCR are divided into CD4+ and CD8+ subsets based on their expression of the co-receptors CD4 and CD8 respectively. CD4+ T cells recognise antigen presented by MHC class II molecules, whereas MHC class I molecules present

antigen to CD8+ T cells. Both CD4+ and CD8+ T cells contribute to the anti-mycobacterial immune response. CD4+ T cells increase within the circulation of calves around 4-6 weeks post-BCG vaccination (Buza et al., 2009) and a similar increase was also evident following infection with *M. bovis* (Pollock et al., 1996). CD4+ T cells also proliferate and produce IFN-γ in response to BCG-infected DCs (Hope et al., 2000a). A major function of CD4+ T cells is as helper lymphocytes and upon antigen recognition, naïve CD4+ T cells can differentiate into various subsets of CD4+ T helper cells. This differentiation is dictated in part by the cytokine milieu present at the time of differentiation and these cytokines are produced by populations of innate immune cells (Zhou et al., 2009). For example, the presence of IL-12 and IFN-γ in the local environment results in the development of a Th1 immune response which are characterised by the production of TNF-α, IL-2 and IFN-γ (Figure 1.5). Th1 CD4+ T cells play a major role in the defence against mycobacteria and *M. tb*-infected DCs bias the polarisation of CD4+ Th1 cells in mice through the secretion of IL-12 (Hickman et al., 2002). The importance of Th1 CD4+ T cell responses during anti-mycobacterial immunity is highlighted by the inability of CD4+ T cell deficient mice to control bacterial growth within the lungs and the enhanced susceptibility of HIV+ individuals to TB due to the absence of Th1 CD4+ T cells (Estaquier et al., 1995, Mogues et al., 2001). Protective immunity against *M. bovis* infection in cattle is also driven by Th1 immune responses which are characterised by early and persistent production of IFN-γ (Buddle et al., 2005). Th17 immune responses have also been implicated during anti-mycobacterial immunity in humans (Jurado et al., 2012), mice (Khader and Cooper, 2008) and cattle (Vordermeier et al., 2009). Expression of IL-17 mRNA was induced after *in vitro* stimulation of PBMCs with PPD-b or Ag85A in both BCG/Ag85A vaccinated cattle and *M. bovis* infected cattle. Furthermore, IL-17 responses to Ag85A in BCG/Ag85A vaccinated animals negatively correlated with disease pathology therefore vaccine-induced IL-17 responses may be protective (Vordermeier et al., 2009). Conversely, increased IL-17 production was associated with pathology in *M. bovis*-infected cattle (Blanco et al., 2011). Further research is required to decipher the key components of the CD4+ T cell response induced by mycobacteria and the specific CD4+ T helper cell associated cytokines which may correlate with protection or disease.

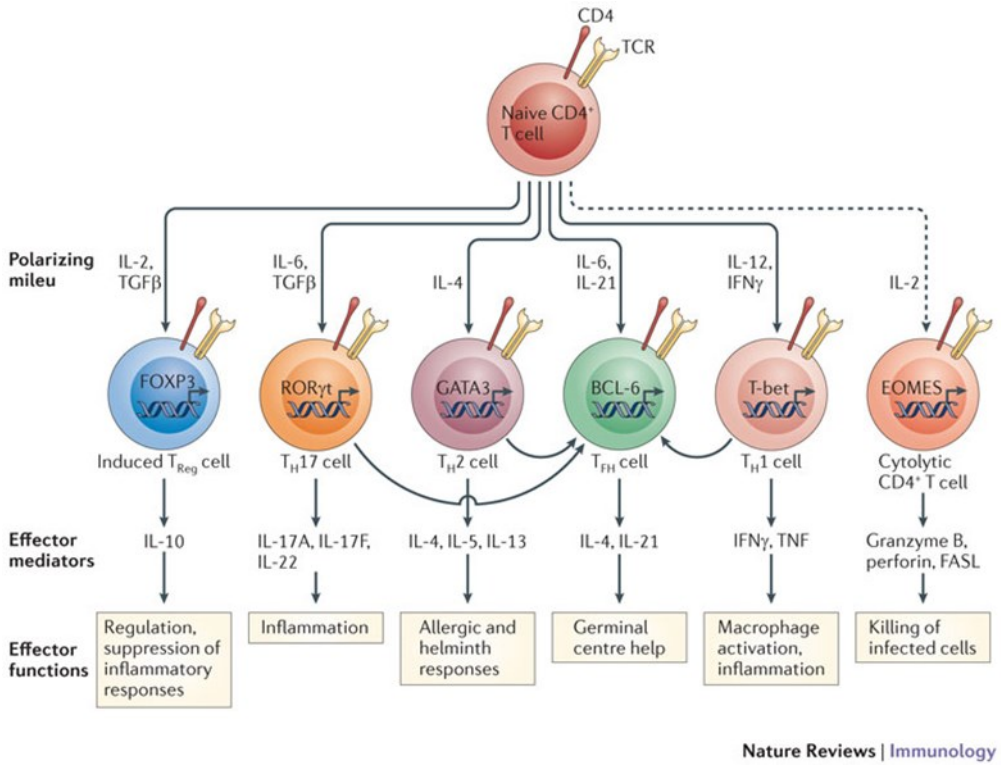


Figure 1.5 Polarisation of CD4+ T helper subsets.

Naïve CD4+ T cells can differentiate into many subsets of T helper cells which is determined in part by the cytokine milieu present at the time of differentiation. These subsets of T helper cells have different effector functions mediated by a wide range of cytokines. Taken from (Swain et al., 2012).

1.6.1.2. CD8+ T cells

CD8+ T cells also contribute to anti-mycobacterial immunity (North and Jung, 2004) and are cytotoxic, proliferate and produce IFN-γ upon activation. CD8+ T cells increase following *M. bovis* infection of calves (Pollock et al., 1996) and the proportion of CD8+ T cells is elevated 8-10 weeks post-BCG vaccination of calves (Buza et al., 2009). Similar to CD4+ T cells, CD8+ T cells produce IFN-γ following BCG vaccination (Hope et al., 2000a). However, *in vivo* depletion of CD8+ T cells prior to challenge with *M. bovis*, resulted in lower lesion scores in the head lymph nodes of depleted calves compared with control calves indicating that CD8+ T cells may contribute to the immunopathology of bTB (Villarreal-Ramos et al., 2003).

1.6.2 B cells

According to the Th1/Th2 paradigm, Th1 cells and Th2 cells protect the host from intracellular and extracellular pathogens respectively (Mosmann and Coffman, 1989). Th2 polarisation of the immune response is driven by the presence of an IL-4 rich cytokine milieu which results in antibody secretion by B cells and production of cytokines including IL-4, IL-5 and IL-13 (illustrated in Figure 1.4). As *Mycobacteria* spp are intracellular pathogens, B cell immune responses were thought not to be important for protective immunity. However, it is become apparent that many diseases elicit mixed cellular and humoral responses, thus challenging the traditional view of the Th1/Th2 paradigm. For example, the protective role of humoral responses to intracellular pathogens such as *Plasmodium*, *Chlamydia, Schistosoma* and *Cryptococcus neoformans* has been established (Abebe and Bjune, 2009).

The importance of antibody in protection of mice and humans following *M. tb* infection of has been demonstrated (Maglione et al., 2007, Lopez et al., 2009). Similarly in cattle, antibodies specific for mycobacteria were present in the sera from 4 weeks post-*M. bovis* infection (Waters et al., 2006a). The presence of B cells within granulomas from *M. bovis*-infected cattle has also been demonstrated, particularly in Stage III and IV granulomas (Aranday-Cortes et al., 2013). Nevertheless, the role of B cells during TB remains controversial.

1.7 Hypothesis and Objectives

Mycobacteria cause significant disease affecting both humans and animals and the incidence of human and bovine TB remains high due to the lack of effective vaccines and associated sensitive and specific diagnostic tests. In order to design better vaccines and induce optimal priming of protection, it is crucial that the immune response elicited by mycobacteria is fully understood. A key to effective immune response induction is the innate immune response and subsequent interactions with the adaptive immune response. NK cells are the focus of this thesis as they are involved during immunisation of infants with BCG (Zufferey et al., 2013) and have an increased frequency and activity in neonatal calves (Kulberg et al., 2004, Graham et al., 2009). Furthermore, they can bridge innate and adaptive immunity through interactions with DCs, which can affect the subsequent adaptive immune response. **The overarching hypothesis being addressed in this thesis is that the enhanced efficacy of BCG in neonatal calves is due to the increased number of NK cells which through interactions with DCs prime Th1-type CD4+ and CD8+ T cell responses.** This project comprised three main objectives which are detailed in Chapters 3, 4 and 5 respectively:

1) Compare the frequency and phenotype of NK cells across peripheral blood, afferent lymph, lymph nodes and efferent lymph in steady-state conditions

2) Determine the effect of BCG vaccination on NK cells derived from peripheral blood, afferent lymph, lymph nodes and efferent lymph

3) Decipher the *in vitro* interaction between NK cells and DCs in the context of BCG

2.1 Media and Suppliers

Details of all media used and suppliers of reagents and equipment are listed in appendix 8.1 and appendix 8.7 respectively.

2.2 Animals

Experiments were performed using male British Holstein-Friesian (*Bos taurus*) calves obtained from the Langhill herd, University of Edinburgh which has been certified free of bTB for over 10 years. All calves were housed at Dryden Farm, University of Edinburgh according to Home Office guidelines and with approval from The Roslin Institute Local Ethics Committee. Calves were under 3 months of age unless otherwise indicated. Age-matched calves were used as controls.

2.2.1 Subcutaneous BCG vaccination

BCG Vaccine Danish strain 1331 was prepared by reconstituting lyophilised BCG with 1ml of diluted Sauton medium. During BCG vaccination experiments, calves were vaccinated with 0.5ml of BCG Danish (5x human dose), administered subcutaneously in the left shoulder. Age-matched control calves were unvaccinated. A sample of the inoculum was plated onto 7H11 agar to determine titre immediately after administration.

Blood samples were collected at various intervals pre- and post-BCG vaccination for up to 12 weeks. To confirm that calves were responding to vaccination, cultures of whole blood were stimulated with PPD-b (10μg/ml) or left unstimulated for 18 hours at 37°C with 5% CO_2. Whole blood cultures were centrifuged at 650g, 4°C for 5 minutes and plasma supernatants were harvested and stored at -20°C prior to analysis. Supernatants were analysed for the presence of IFN-γ by Enzyme Linked Immunosorbent Assay (ELISA) (section 2.15).

To study *in vivo* NK cell responses within skin draining afferent lymphatic vessels (section 2.2.2) in the context of BCG vaccination, 0.5ml of reconstituted BCG Danish

was delivered subcutaneously to the left shoulder of 6 month old pseudo-afferent lymphatic cannulated calves and afferent lymph was collected at various time points pre- and post-vaccination. To assess recruitment of NK cells to the lymph nodes draining the site of vaccination *in vivo*, 0.5ml of reconstituted BCG Danish was administered subcutaneously to the left shoulder of 6 month old calves and prescapular lymph nodes (PSLN) were then excised 24 or 48 hours post-vaccination. PSLN from the right shoulder (non-vaccinated) of each calf served as an internal control.

2.2.2 Afferent Lymphatic Cannulation

Surgical cannulation of skin draining afferent lymphatic vessels of male British Holstein-Friesian (*Bos taurus*) calves aged 3-6 months old was kindly performed by Dr Charlotte Bell using a protocol described in detail elsewhere (Hope et al., 2006) and as illustrated in Figure 2.1. Briefly, prior to cannulation of the afferent lymphatic vessels, the left and right PSLNs were identified and excised. Approximately 8 weeks later, following re-anastomosis of the small afferent lymphatic vessels to the larger efferent lymphatic vessel, a sterilised surgical cannula was inserted into this 'pseudo-afferent' lymphatic vessel. Lymph was collected into a T75 tissue culture flask, which was secured to the side of the calf using a harness, and contained 1ml of sodium benzylepenicillin dissolved in heparin (3g sodium benzylepenicillin dissolved in 5000i.u/ml heparin). Flasks were changed twice daily, or as required, for up to 28 days post-cannulation. Paired samples of peripheral blood were collected by jugular venepunture into 10ml vacutainers containing sodium heparin (10U/ml). PBMCs were then isolated as detailed in section 2.3.1.

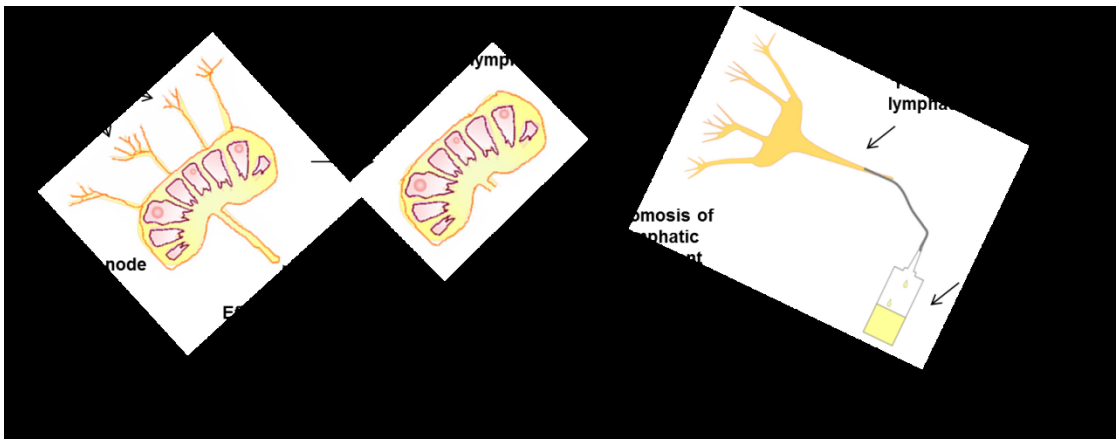


Figure 2.1 Illustration represents the steps involved to cannulate the skin draining afferent lymphatic vessels.

To cannulate the 'pseudo-afferent' lymphatic cannulation vessel, the lymph node was excised and approximately 8 weeks later following re-anastomosis of the small afferent lymphatic vessels to the larger efferent lymphatic vessel, a cannula was inserted into the 'pseudo-afferent' lymphatic vessel. Lymph was then be collected into a flask which was attached to the side of the calf and contains cells which are trafficking to lymph nodes via the afferent lymphatic vessel. Adapted from (Neeland et al., 2014).

2.2.3 Efferent Lymphatic Cannulation

Samples of frozen efferent lymph from naïve efferent lymphatic cannulated calves were kindly provided by Dr Bernardo Villareal-Ramos (APHA). Efferent lymphatic vessels of male British Holstein-Friesian (*Bos taurus*) calves aged 3-6 months old were surgically cannulated by Dr Bryan Charleston using a method described previously (Vrieling et al., 2012). Following surgical cannulation, efferent lymph was collected into flasks containing sodium benzylepenicillin dissolved in heparin as described in section 2.2.2. Paired samples of peripheral blood were collected by jugular venepunture into 10ml vacutainers containing sodium heparin (10U/ml). PBMCs were then isolated as detailed in section 2.3.1. Three efferent lymphatic cannulated calves (aged 6 months) were vaccinated subcutaneously with 0.5ml reconstituted BCG Danish and efferent lymph was collected at various time points pre- and post-vaccination.

2.3 Isolation of cells

2.3.1 Peripheral blood mononuclear cells (PBMCs)

50ml volumes of blood were collected by jugular venepuncture into syringes containing sodium heparin (10U/ml) or for larger blood volumes (between 300-500ml), blood was collected into bags containing citrate phosphate dextrose adenine (CPDA), and PBMCs were separated by density gradient centrifugation. Depending on the age of the calf, blood was diluted either 1:2 (if less than 3 months old) or 1:3 with phosphate buffered saline (PBS) and layered onto Histopaque 1083. Gradients were centrifuged at 1200g, 20°C for 35 minutes with the brake disabled and the buffy coat containing PBMCs was retrieved. Cells were washed three times with PBS, once at 650g, 4°C for 10 minutes and twice at 400g, 4°C for 8 minutes. Contaminating erythrocytes were removed by adding 3ml of 0.1M ammonium chloride lysis buffer for 5 minutes followed by washing with PBS at 400g, 4°C for 8 minutes. Viable cells were counted using trypan blue exclusion in a haemocytometer and PBMCs were resuspended in an appropriate volume of tissue culture medium (TCM) prior to use. Fresh cells were analysed by flow cytometry.

2.3.2 Mononuclear cells (MNCs) from PSLNs

PSLNs were collected into ice cold PBS, sliced in half, and inner regions were scored using a scalpel to aid removal of the cells from the tissue. Cells were gently teased away from the capsule using a blunt scalpel, collected into PBS and filtered through a sterile 40μM cell strainer. The cell suspension was diluted in PBS and MNCs were obtained by density gradient centrifugation using Histopaque 1083 (described in detail in section 2.3.1). MNCs were resuspended in TCM and viable cells were counted using trypan blue exclusion in a haemocytometer. Fresh cells were analysed by flow cytometry.

2.3.3 MNCs from afferent lymph

Whole lymph was centrifuged at 400g, 4°C for 5 minutes to pellet the cells. Cells were then resuspended in TCM and viable cells were counted using trypan blue exclusion in a haemocytometer. Fresh cells were analysed by flow cytometry.

2.3.4 MNCs from the skin

Small sections of shaved skin were digested in RPMI containing dispase I (20μg/ml) and collagenase (75U/ml) for 90 minutes at 37°C in a shaking incubator. The digest was then homogenised and filtered through a 40μM cell strainer. Cells were centrifuged at 400g, 4°C for 5 minutes to pellet cells, resuspended in TCM and viable cells (1×10^6) were counted using trypan blue exclusion in a haemocytometer. Fresh cells were analysed by flow cytometry.

2.3.5 CD14+ monocytes and culture of monocyte-derived DCs

MACS MicroBeads conjugated to a mouse anti-human CD14 antibody, which has been shown to be cross-reactive with bovine monocytes (Werling et al., 1999) were used to isolate CD14+ monocytes. PBMCs were incubated with 2.5μl beads/10^7 cells for 10 minutes at room temperature. Following labelling of PBMCs, cells were washed twice with PBS at 400g, 4°C for 5 minutes and resuspended with 2.5ml FACSFlow/1% Bovine Serum Albumin (BSA). CD14+ monocytes were positively selected using a MidiMACS LS column and washed twice with PBS (400g for 5 minutes at 4°C).

CD14+ monocytes were diluted to 1 x 10^6 cells/ml in TCM and 3 x 10^6 monocytes were seeded in 6-well plates with a 1:300 dilution of COS cell derived recombinant bovine GM-CSF and IL-4 (generated as detailed in section 2.16). CD14+ monocytes were incubated for 3 days at 37°C with 5% CO_2 to obtain immature monocyte-derived DCs. All monocyte-derived DCs were used fresh.

2.3.6 NK cells

PBMCs were diluted to 5 x 10^7 cells/ml in PBS/2mM ethylenediaminetetraacetic acid (EDTA)/0.5% BSA and 3µg/ml of mouse anti-ovine NKp46 (clone EC1.1, IgG1) was added. PBMCs were rolled gently for 30 minutes at 4°C, washed twice with PBS/2mM EDTA/0.5% BSA (400g for 10 minutes at 4°C) and resuspended to 5 x 10^7 cells/ml in PBS/2mM EDTA/0.5% BSA. Immunomagnetic pan-mouse IgG Dynabeads were prepared by washing the required volume (10µl beads per 10^7 cells) with 1ml PBS/2mM EDTA/0.5% BSA. Beads were added directly to labelled PBMCs diluted in PBS/2mM EDTA/0.5% BSA and rolled for a further 30 minutes at 4°C. The volume of cell suspension was doubled with PBS/2mM EDTA/0.5% BSA and transferred to 15ml falcon tubes. Falcon tubes were placed in a DynaMag-15 for 2 minutes and unbound cells were poured off. NKp46+ cells were washed a further 3 times with 5ml PBS/2mM EDTA/0.5% BSA, removed from the magnet and resuspended in TCM. 5 x 10^5 cells/ml were seeded in 24-well plates and incubated at 37°C with 5% CO_2. NK cells were harvested from the plates 24 hours later, mixed thoroughly and placed in a DynaMag-15 for 2 minutes to remove the magnetic beads. Resulting NK cells were collected and washed once with PBS (400g for 5 minutes at 4°C), resuspended in TCM and counted using trypan blue exclusion and a haemocytometer. All NK cells were used fresh and an average of 2.5 x 10^6 cells were obtained from 300-500ml of blood.

2.3.7 Cryopreservation

Cells were frozen by resuspending cells at 5 x 10^7 cells/ml in FCS containing 10% dimethyl sulfoxide (DMSO). 1ml volumes were then transferred into cryovials and placed in a freezing container containing isopropanol and stored at -80°C. Aliquots were thawed immediately after removal from the freezer using a 37°C water bath and washed twice with PBS prior to use.

2.4 Labelling BCG with FITC

BCG was reconstituted as previously described (section 2.2.1), mixed with an equal volume of 0.2mg/ml FITC and incubated for 3 hours at 4°C, protected from light. Bacteria were washed with PBS at 800g, 4°C for 20 minutes and this was repeated 3 times. Fluorescently labelled bacteria were then resuspended in an appropriate volume of RPMI + 10% Foetal Bovine Serum (FBS) prior to infection of DCs (section 2.5).

2.5 Infection of DCs with BCG/BCG-FITC

Immature monocyte-derived DCs were harvested by firstly removing floating cells which were stored on ice prior to combining with adherent cells. Adherent cells were incubated with 1ml non-enzymatic cell dissociation fluid for 30 minutes at 37°C with 5% CO_2. Floating and adherent cells were pooled and washed once with PBS (400g for 5 minutes at 4°C) and resuspended in RPMI + 10% FCS. BCG was reconstituted as described (section 2.2.1), washed once with PBS at 400g, 4°C for 5 minutes and resuspended in RPMI + 10% FCS. DCs were pelleted and resuspended in 1ml of BCG or BCG-FITC at a multiplicity of infection (MOI) of 5 for 24 hours at 37°C with 5% CO_2. This MOI was selected after determining the percentage of DCs that took up BCG-FITC and the viability of DCs after infection of DCs with BCG at MOIs of 1, 3 and 5 (Figure 5.2.2 and 5.2.3).

2.6 Exposure of DCs to Zylexis™

Zylexis™ is an immunomodulatory compound produced by the industrial partner of this PhD project, Zoetis. It is used to non-specifically stimulate the immune response of horses therefore it was tested within co-cultures of bovine NK cells and monocyte-derived DCs to determine if it could act as an immunomodulator in this context. Immature DCs were harvested as described in section 2.5. Zylexis™ was reconstituted in distilled water, washed once with PBS at 400g, 4°C for 5 minutes and resuspended in RPMI + 10% FCS. DCs were pelleted and resuspended in 1ml of Zylexis™ at a multiplicity of infection (MOI) of 1 for 24 hours at 37°C with 5% CO_2. This MOI was defined by determining cell viability after exposing DCs to Zylexis™ at MOIs of 1, 5 and 10.

2.7 Co-culture of NK cells with BCG-infected DCs

BCG-infected DCs or uninfected DCs were diluted to 4 x 10^5 cells/ml in RPMI + 10% FCS and seeded in a 96 well 'U' bottom plate at 100µl cells per well for 24 hours at 37°C with 5% CO_2. NK cells were diluted to 2 x 10^6 cells/ml in RPMI + 10% FCS and added to the wells containing DCs at 100µl cells per well (at a previously determined optimal NK cell : DC ratio of 5:1 (Siddiqui and Hope, 2012)). NK cells and DCs were incubated for 18 hours at 37°C with 5% CO_2. Following co-culture, cells were pelleted and supernatants were collected and stored at -20°C prior to analysis of cytokine production by ELISA (section 2.15). Expression of NKp46, CD3, CD2, CD25 and NKp30 by NK cells, and MHC class I, MHC class II, CD40, CD80, CD86 and CCR7 by DCs were analysed using multi-colour flow cytometry (as described in section 2.13). NK cells cultured alone, with BCG (MOI of 5) or supplemented with recombinant bovine IL-12 (20BU/ml) and recombinant human IL-18 (20ng/ml) were the control conditions.

2.8 Co-culture of NK cells with Zylexis™-exposed DCs

Co-cultures of NK cells and Zylexis™-exposed DCs were performed as described in section 2.7.

2.9 RNA extraction

Prior to RNA extraction, 1 x 10^6 isolated NK cells were lysed using RLT buffer containing 2-mercaptoethanol and stored at -80°C. Lysed cells were thawed and homogenised using a Qiashredders column. RNA was extracted using RNeasy mini kits, which included a DNase digestion step to remove contaminating genomic DNA, according to the manufacturer's instructions. RNA was eluted into RNase-free water and the quality and concentration of the RNA was assessed using the Nanodrop spectrophotometer ND-1000. RNA was stored at -80°C until use.

2.10 Reverse transcription

RNA was reverse transcribed using the Superscript III Reverse Transcriptase system according to the manufacturer's instructions. The quality of cDNA was assessed by

detecting the presence of the housekeeping gene GAPDH by PCR (primers illustrated in appendix 8.4).

2.11 Polymerase Chain Reaction (PCR)

To assess the expression of *KLRC1.1*, *KLRC2.1* and *KLRC2.2* by isolated NK cells at various time points post-BCG vaccination, PCR reactions for were performed using the primers illustrated in appendix 8.4. Mastermixes (same composition for each gene) contained: 1µl cDNA, 1.25µl forward and 1.25µl reverse primers, 0.5µl dNTPs, 0.25µl Taq polymerase, 2.5µl 10x PCR reaction buffer, 1.25µl $MgCl_2$ and reactions were made up to a volume of 25µl with distilled water. Reactions were run according to the programmes illustrated below using cDNA positive for the gene of interest as the positive control and water as the negative control.

95°C 2 minutes

95°C 30 seconds
56°C 30 seconds
72°C 1 minute
} x 35 cycles

72°C 7 minutes

PCR products were mixed with 5µl loading dye and loaded on a 2% agarose gel containing 10.5µl SYBR Safe at 115 volts (V) for 2 hours. 5µl of 1Kb PLUS DNA ladder diluted 1:5 with loading dye was run alongside the PCR products to define size. Expected band sizes for each gene were as follows: *KLRC1.1* (140bp), *KLRC2.1* (140bp) and *KLRC2.2* (180bp).

2.12 Quantitative real – time PCR

To assess expression of *NKp30* (*NCR3*) by NK cells prepared at various time points pre- and post-BCG vaccination and NK cells from age-matched, non-vaccinated control calves, qPCR assays were performed for these target genes alongside the reference genes, *ATP5B* and *EIF2B2.* These reference genes were decided as the most suitable after testing five reference genes (Alasdair Allan, PhD thesis, Royal

Veterinary College, 2015). Primer sequences are illustrated in appendix 8.5. Mastermixes (compositions detailed in appendix 8.5) were prepared for each gene and contained: Luminaris Colour HiGreen Low ROX qPCR master mix, cDNA, forward primer, reverse primer and were made up to a volume of 20µl with distilled water. Each sample was tested in duplicate. qPCR was performed using the Viia 7 Real Time PCR system with cDNA positive for the gene of interest as the positive control, cDNA amplified with Taq polymerase as the reverse transcription control and water as the negative control.

2.13 Multi-colour immunofluorescent staining

Flow cytometry of cell surface molecules was performed on PBMCs, MNCs, NK cells, and DCs. For 4 colour direct/indirect staining, 10^6 cells were added to wells of a 96 well 'U' bottom plate and centrifuged at 650g for 2 minutes. Primary antibodies, diluted in PBS/1% BSA/0.1% sodium azide were added and cells were incubated for 15 minutes at room temperature, protected from light. Following two washes with PBS/1% BSA/0.1% sodium azide (650g for 2 minutes); fluorochrome-labelled isotype-specific secondary antibodies were added for 15 minutes in the dark. After one wash with PBS/1% BSA/0.1% sodium azide (650g for 2 minutes), 10% normal mouse serum was added to cells for 15 minutes to prevent non-specific binding. Lastly, one or two directly conjugated antibodies were added and after a final two washes at 650g for 2 minutes, cells were resuspended in FACSFlow. An equal volume of Sytox Blue, to discriminate live cells, was added prior to visualisation and collection of 50,000 events using the BD LSRFortessa and FACSDiva Software. Data was analysed using FlowJo v10 software. Appendix 8.2 and 8.3 illustrate the mAbs and fluorochromes used respectively.

2.14 Intracellular cytokine staining

10^7 PBMCs/MNCs were resuspended in RPMI 1640 + 10% FCS with 20 x PMA/Ionomycin/Brefeldin A and incubated for 4 hours at 37°C with 5% CO_2. Following stimulation, Zombie Aqua Fixable Viability Kit was added at a 1:200 dilution in 100ul PBS per 10^6 cells for 15 minutes protected from light. PBMCs were washed twice with PBS and fixed by the addition of 2% paraformaldehyde for 30

minutes at room temperature. Cells were then washed with PBS and permeabilised by adding 1x Permeabilisation Solution. Cells were resuspended overnight in PBS/1% BSA/0.1% sodium azide prior to staining of surface and intracellular molecules.

2.15 Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were performed to detect the presence of bovine IL-12 (Hope et al., 2002a) and IFN-γ (Collins et al., 1999). Appendix 8.6 illustrates the capture antibodies, detection antibodies and cytokine standards used. 96-well transparent, flat-bottomed plates were coated with capture antibody diluted in carbonate/bicarbonate buffer, sealed and left overnight at room temperature. Following six washes with PBS/Tween-20 using the Skan Washer 400 plate washer, non-specific binding of the antibody was blocked by adding 1mg/ml sodium casein in PBS for 1 hour at room temperature. Plates were then washed six times with PBS/Tween-20. Recombinant cytokines were serially diluted (2-fold for IFN-γ and 3-fold for IL-12) and 100µl of each dilution and 100µl of sample were plated in duplicate and incubated for 1 hour at room temperature. Plates were washed a further six times with PBS/Tween-20 and incubated for 1 hour with biotinylated detection antibody, diluted to the appropriate concentration in 1mg/ml sodium casein in PBS. Following additional washing, Streptavidin-HRP Peroxidase, diluted 1:500 with 1mg/ml sodium casein/Tween-20, was added for 45 minutes at room temperature. After a final six washes, the plates were developed in the dark using 100µl/well of 3,3´,5,5´-tetramethylbenzidine (TMB) substrate solution and the reaction was stopped using 50µl/well of 1M sulphuric acid. Absorbance was measured at 450nm subtracted from 690nm using the Synergy HT Multi-Mode Microplate Reader and Gen 5 software.

2.16 Generating recombinant bovine GM-CSF and IL-4

Recombinant bovine GM-CSF and IL-4 were obtained from stably-transfected Chinese Hamster Ovary (CHO) cell lines, which were kindly provided by Professor Gary Entrican and Sean Wattegedera (Moredun Research Institute). Cloned lines of transfected CHO cells were maintained in Glasgow's Modified Eagle's Medium (GMEM) supplemented with sodium pyruvate, non-essential amino acids and USDA-approved dialysed foetal calf serum (FBS). For cytokine production, CHO cells were

cultured in FBS-free GMEM and supernatants containing GM-CSF or IL-4 were collected and stored at -20°C (Entrican et al., 1996).

2.17 Data analysis

Data analysis was performed using Microsoft Excel 2010 and GraphPad Prism 6. Statistical analysis was completed using Minitab v16. Distribution of data was assessed using a normality test with data with $p<0.05$ deemed to be of a non-normal distribution. Non-normal data was log transformed. Statistical methods used are detailed in individual figure legends and included paired t test, 2-sample t test and generalised linear models (GLM). $p<0.05$*, $p<0.01$** and $p<0.001$***.

Abstract

NK cells are widely distributed in lymphoid and non-lymphoid tissues however little is known about the recirculation of NK cells between blood and tissues. Therefore, the percentage of NK cells and their phenotype across peripheral blood, afferent lymph and lymph nodes in steady-state conditions was investigated using the pseudo-afferent lymphatic cannulation model. CD2+ $CD25^{lo}$ NK cells were the predominant subset of NK cells within the blood. In contrast, CD2- $CD25^{hi}$ NK cells were the main subset present within the skin draining afferent lymphatic vessels and lymph nodes indicating that CD2- NK cells are the principal NK cell subset trafficking to LNs via the afferent lymphatic vessel. Furthermore, NK cells were present in efferent lymph and therefore suggests that NK cells, particularly the CD2- subset, can egress from lymph nodes and return to circulation in steady-state conditions. Thus, NK cells may represent a population of recirculating lymphocytes in steady-state conditions.

3.1 Introduction

NK cells are highly motile lymphocytes that are widely distributed in lymphoid and non-lymphoid tissues (Gregoire et al., 2007, Connelley et al., 2011, Boysen et al., 2008, Mair et al., 2012, Tomasello et al., 2012) allowing them to respond quickly to infection or inflammation through cytotoxicity and production of immunoregulatory cytokines. Dissecting the precise anatomical location of NK cells will reveal potential roles for these cells in steady-state and inflammatory conditions. Furthermore, NK cells interact with an array of accessory cell populations including DCs and therefore, it is important to decipher where these interactions may take place *in vivo*, as such innate immune cell interactions are pivotal in driving the nature of the adaptive immune response.

Lymphocytes continuously circulate between the blood and tissues such as the skin via the lymphatics and lymph nodes in steady-state conditions. This continuous recirculation is required for immune surveillance (Gowans, 1959). Lymphocytes can enter lymph nodes via two routes, firstly from the circulation through high endothelial venules (HEVs) and secondly, they can migrate from the tissues through afferent lymphatic vessels. Lymphocytes can then egress from the lymph nodes via the efferent lymphatic vessel and return to circulation via the thoracic duct (Masopust and Schenkel, 2013). In contrast to the re-circulatory nature of αβ T cells, very little is known about NK cell recirculation (Carrega and Ferlazzo, 2012). It has been demonstrated in mice that NK cells migrate from the circulation to lymph nodes via HEVs (Martin-Fontecha et al., 2004, Bajenoff et al., 2006). NK cells are present in human afferent lymphatic vessels (Yawalkar et al., 2000, Hunger et al., 1999, Carrega et al., 2014) and to date, one study has shown that NK cells are found in human efferent lymph (Romagnani et al., 2007). It was demonstrated recently that bovine NK cells are present in the skin draining afferent lymphatic vessels of cattle in steady-state conditions. In this study, the frequency, phenotype and function of NK cells from

peripheral blood and afferent lymph of different animals were compared using the pseudo-afferent lymphatic cannulation model (Lund et al., 2013).

Surgical cannulation of afferent and efferent lymphatic vessels has been used to study innate immune responses during vaccination and infection in animal models. In contrast to efferent lymphatic vessels, afferent lymphatic vessels are difficult to surgically access, even in large animals, therefore to access cells trafficking through skin draining afferent lymphatic vessels, the pseudo-afferent lymphatic cannulation model can be utilised as previously described (Hope et al., 2006) and as detailed in Figure 2.1. Briefly, approximately eight weeks prior to cannulation of the afferent lymphatic vessels, the left and right PSLNs are identified and excised. Following re-anastomosis of the small afferent lymphatic vessels to the larger efferent lymphatic vessel, which takes place over a period of six-eight weeks, a sterilised surgical cannula is inserted into this 'pseudo-afferent' lymphatic vessel, to allow collection of afferent lymph that contains cells which would have been migrating into the lymph nodes *in vivo*. This method is well established in cattle, sheep, pigs and rats (Hope et al., 2006, Schwartz-Cornil et al., 2006, Johnson and Jackson, 2008, Liu et al., 1998).

Many vaccines (including BCG) are delivered subcutaneously. Our knowledge of why vaccines work whereas others fail at a mechanistic level remains limited. Priming of the adaptive immune response in mammals occurs in organised lymphoid tissue, hence investigation of the local immune response occurring at the site of vaccination and in the draining lymph nodes will help improve vaccine design and delivery. The work presented in this Chapter assessed the frequency and phenotype of bovine NK cells in steady-state conditions across peripheral blood (PB), afferent lymph (AL) and lymph nodes (LNs). To do this, the pseudo-afferent lymphatic cannulation model was utilised, with a total of seven calves. LNs were analysed at the time of excision, and PB and AL from the same animal was collected post-surgical cannulation and analysed in parallel. Furthermore, samples of efferent lymph (EL) from five naïve efferent lymphatic cannulated calves were kindly provided by Dr Bernardo Villareal-Ramos (APHA) to determine if NK cells were present in bovine EL.

The hypothesis of this study was that the frequency and phenotype of bovine NK cells in steady-state conditions is reflective of their anatomic localisation. Understanding the frequency and phenotype of innate lymphocytes such as NK cells will inform on the design of appropriate vaccines/adjuvants/immunomodulators and will also help to decipher the innate interactions that drive the bias of the adaptive immune response.

3.2 Results

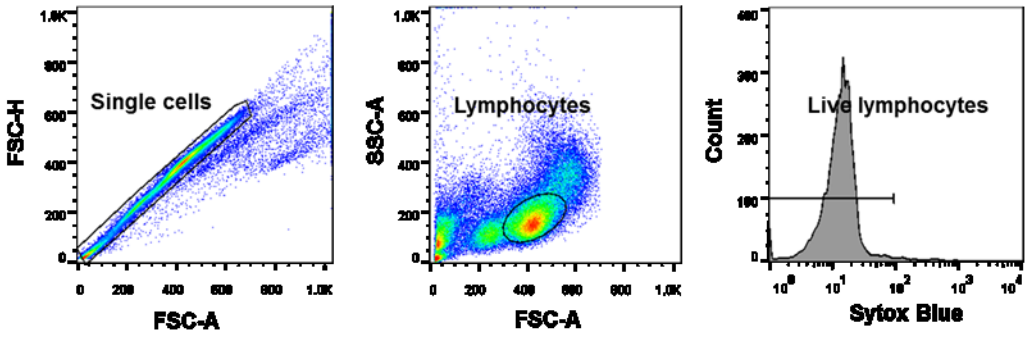
3.2.1 Percentage of NK cells present in PB, AL and LNs

Firstly, to determine if NK cells were present in skin draining afferent lymphatic vessels and at what frequency compared with those present in PB and LNs, the abundance of NKp46+ CD3- NK cells within PB, AL and LNs of seven calves was investigated. Lymphocytes were labelled with mAbs to NKp46 and CD3 and analysed by flow cytometry. The gating strategy used to identify lymphocytes throughout this thesis is illustrated in Figure 3.1.1. NKp46+ CD3- NK cells represented 5.1% (2.05 - 10.3%; SD = 3.1), 4.8% (1.39 - 7.68%; SD = 1.9), and 6% (3.03 - 11.7%; SD = 2.9) of lymphocytes in PB, AL and the LNs respectively. No significant differences were evident between the percentages of NK cells present across the three compartments (Figure 3.1.3).

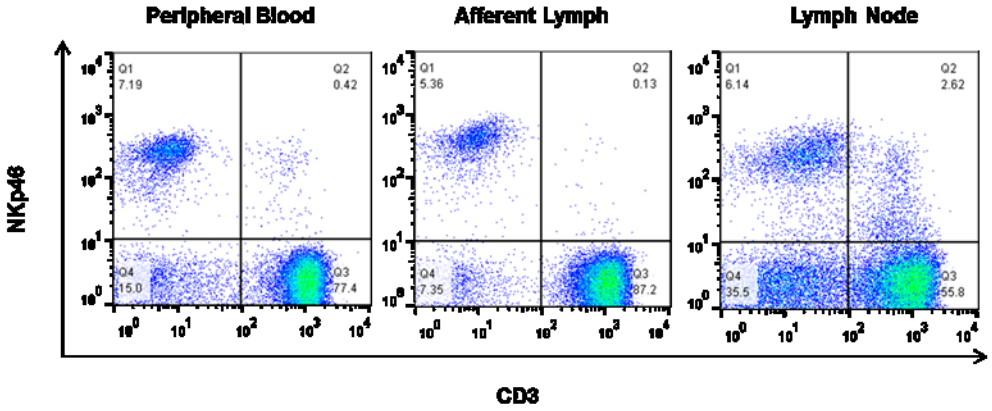
3.2.2 NK cell subsets present in PB, AL and LNs

Similar to human (Cooper et al., 2001a) and murine (Chiossone et al., 2009) NK cells, bovine NK cells exist as distinct subsets and consequently NKp46+ NK cells can be subdivided into two subsets based on their differential expression of CD2 (Boysen et al., 2006). Therefore, to investigate the distribution of CD2+ and CD2- subsets of NK cells across PB, AL and LNs, lymphocytes were labelled with mAbs to NKp46 and CD2 and analysed by flow cytometry. CD2+ NK cells were the predominant subset in PB; however CD2- NK cells were the principal NK cell subset found in the skin draining afferent lymphatic vessels and LNs. Furthermore, CD2- NK cells were present in AL ($p<0.001$) and LNs ($p<0.001$) at a significantly higher proportion compared with PB (Figure 3.2.2).

3.1.1


Single cells
FSC-H
FSC-A
Lymphocytes
SSC-A
FSC-A
Live lymphocytes
Count
Sytox Blue

3.1.2


Peripheral Blood
Afferent Lymph
Lymph Node
NKp46
CD3
Q1 7.19
Q2 0.42
Q4 15.0
Q3 77.4
Q1 5.36
Q2 0.13
Q4 7.35
Q3 87.2
Q1 6.14
Q2 2.62
Q4 35.5
Q3 55.8

3.1.3

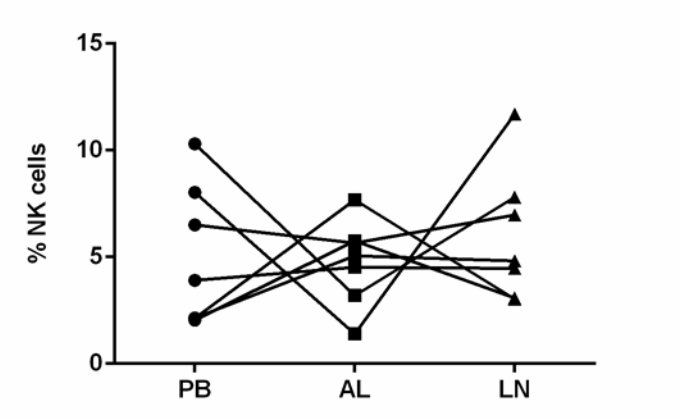

% NK cells
15
10
5
0
PB
AL
LN

Figure 3.1 Percentage of NK cells present in PB, AL and LNs

Lymphocytes derived from PB (fresh), AL (fresh) and the LNs (fresh) of seven calves were labelled with mAbs to NKp46 and CD3 and analysed by flow cytometry. Single cells were gated, followed by lymphocytes and then live lymphocytes were selected as those cells which were negative for the dead cell discriminator, Sytox Blue (Figure 3.1.1). FACS plots from one representative animal illustrate the expression of NKp46 and CD3 by PB, AL and LN derived lymphocytes (Figure 3.1.2). Quadrants were set based on Fluorescence Minus One (FMO) controls. Pooled data from seven calves indicate the percentage of NKp46+ CD3- NK cells present in PB (circles), AL (squares) and LNs (triangles) (Figure 3.1.3). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. No significant differences were noted.

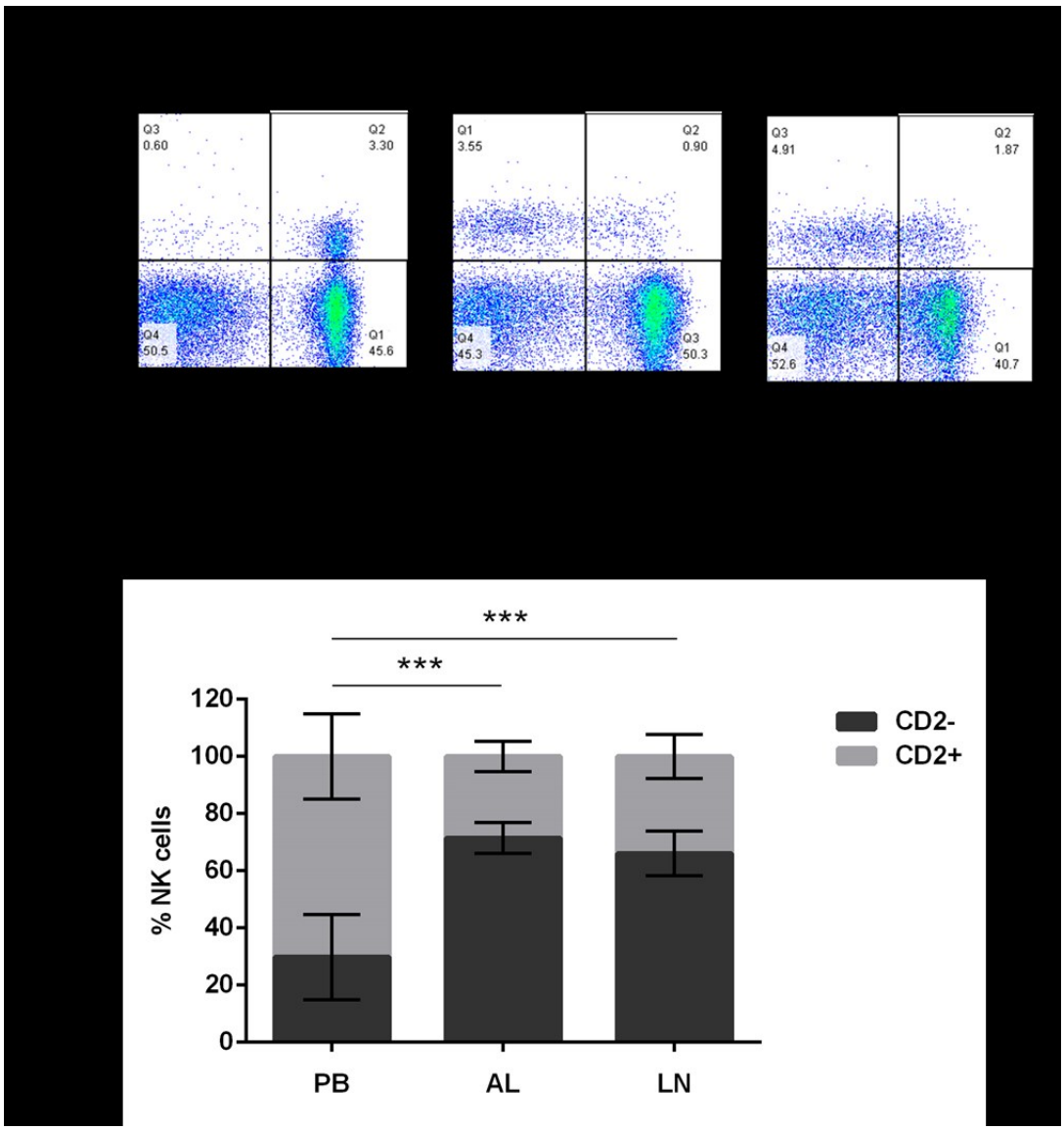


Figure 3.2 NK cell subsets present in PB, AL and LNs

Lymphocytes derived from PB (fresh), AL (fresh) and the LNs (fresh) of seven calves were labelled with mAbs to NKp46 and CD2 and analysed by flow cytometry. FACS plots from one representative animal illustrate the expression of NKp46 and CD2 by PB, AL and LN derived lymphocytes (Figure 3.2.1). Quadrants were set based on FMO controls. Pooled data from seven calves illustrates the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population from PB, AL or the LNs (Figure 3.2.2). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.001$***.

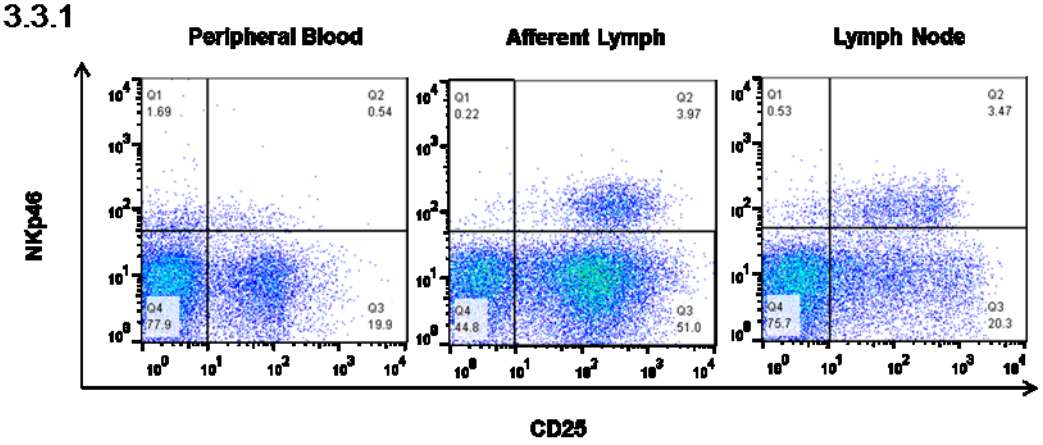
3.2.3 Activation of NK cells derived from PB, AL and LNs

The activation status of NK cells derived from PB, AL and the LNs was analysed by determining the expression of the activation marker, CD25 which is the α-chain of the IL-2 receptor. Lymphocytes were labelled with mAbs to NKp46, CD2 and CD25 and analysed by flow cytometry. In contrast to PB where 26.3% (13.3 - 46.2%; SD = 10.9) of NK cells were CD25+, the percentage of CD25+ NK cells was significantly increased in AL ($p<0.001$) and LNs ($p<0.001$), with 85.8% (66.1 - 95.2%; SD = 12.2) and 81.4% (74.8 - 87.9%; SD = 5.1) of AL and LN derived NK cells expressing CD25 respectively (Figure 3.3.2). A similar increase in the expression levels of CD25, represented by Mean Fluorescence Intensity (MFI), was also noted and CD25 expression was significantly enhanced on AL ($p=<0.001$) and LN ($p=0.013$) derived NK cells, compared with NK cells from PB (Figure 3.3.3). To define the subset of NK cells responsible for the increased activation of NK cells in AL and LNs, the percentage of CD2+ and CD2- NK cells within the CD25+ population from PB, AL and LNs was assessed. CD25 was equally expressed by PB derived CD2+ and CD2- NK cells, however the percentage of CD2- CD25+ NK cells increased significantly in AL ($p<0.001$) and the LNs ($p=0.005$) (Figure 3.3.4).

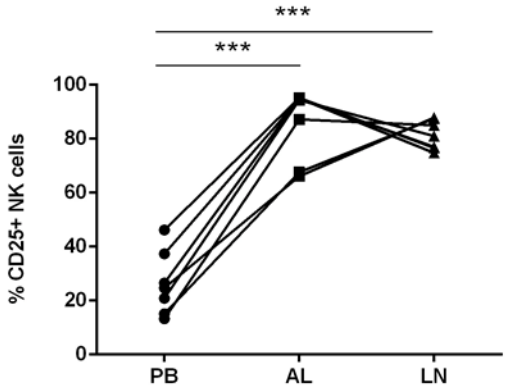
3.2.4 Migratory potential of NK cells present in PB, AL and LNs

To assess the migratory potential of NK cells, the expression of the LN homing molecules CD62L (L-selectin) and CCR7 were assessed by PB, AL and LN derived NK cells. Lymphocytes were labelled with NKp46, CD2, CD62L and CCR7 and analysed by flow cytometry. No significant differences between the percentages of CD62L+ NK cells present across PB, AL and LNs were observed (Figure 3.4.2). However, the percentage of CD2- NK cells expressing CD62L was significantly increased in AL ($p=0.004$) and LNs ($p=0.024$), compared with PB (Figure 3.4.3). CCR7 expression was not detected on PB and LN derived NK cells and the expression was highly variable on AL derived NK cells (Figure 3.4.4).

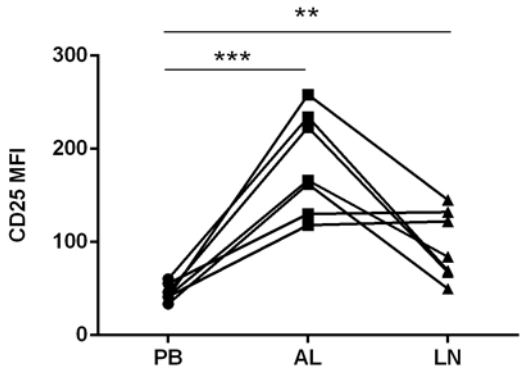
3.3.1



3.3.2



3.3.3



3.3.4

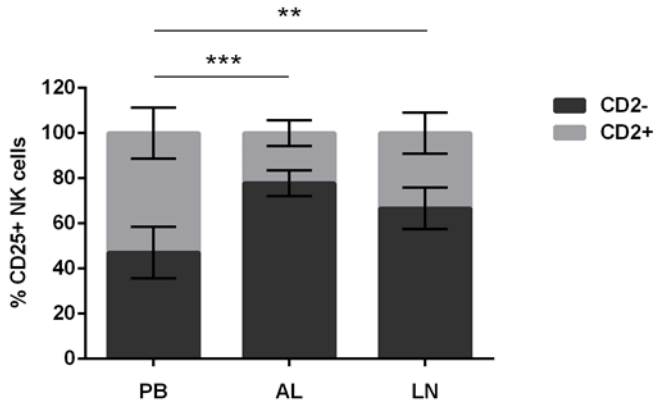
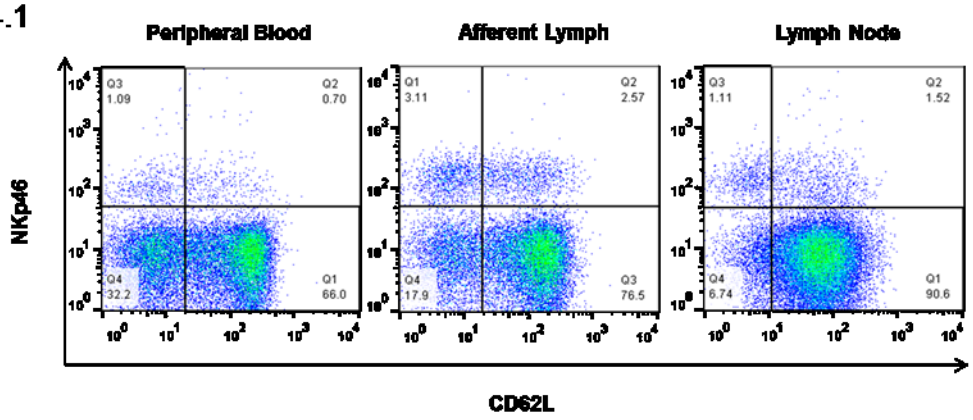


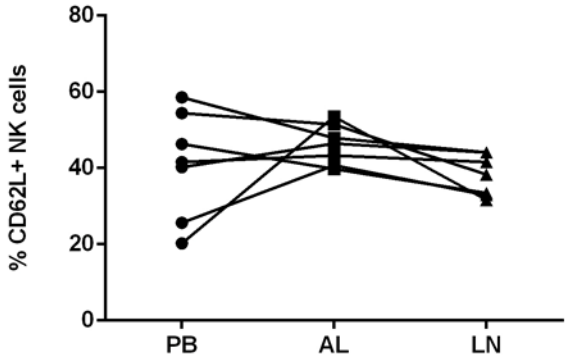
Figure 3.3 Activation of NK cells derived from PB, AL and LNs

Lymphocytes from PB (fresh), AL (fresh) and the LNs (fresh) of seven calves were labelled with mAbs for NKp46, CD2 and CD25 and analysed by flow cytometry. FACS plots from one representative animal illustrate the expression of NKp46 and CD25 by PB, AL and LN derived lymphocytes (Figure 3.3.1). Quadrants were set based on FMO controls. Pooled data from seven calves illustrates the percentage of CD25+ NK cells within the total gated NKp46+ NK cell population from PB (circles), AL (squares) or the LNs (triangles) (Figure 3.3.2). Pooled data from seven calves indicates the MFI of CD25+ NK cells within the total gated NKp46+ NK cell population from PB (circles), AL (squares) or the LNs (triangles) (Figure 3.3.3). Pooled data from seven calves illustrates the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ CD25+ population from PB, AL or LNs (Figure 3.3.4). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.01$**, $p<0.001$***.

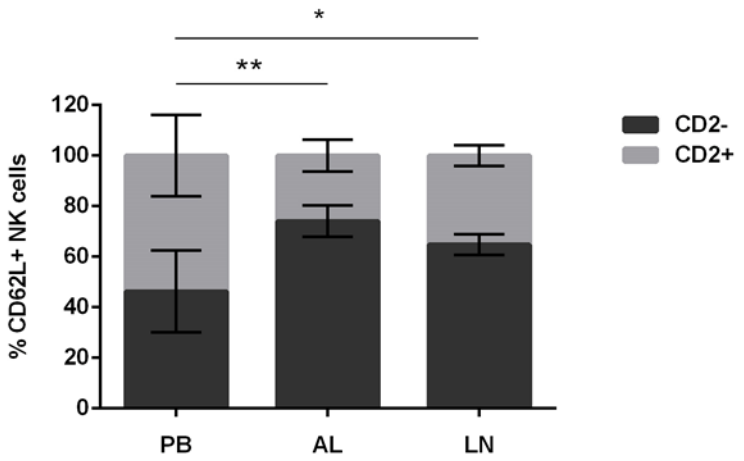
3.4.1


Peripheral Blood
Afferent Lymph
Lymph Node
NKp46
CD62L
Q3 1.09
Q2 0.70
Q4 32.2
Q1 66.0
Q1 3.11
Q2 2.57
Q4 17.9
Q3 76.5
Q3 1.11
Q2 1.52
Q4 6.74
Q1 90.6

3.4.2


% CD62L+ NK cells
80
60
40
20
0
PB
AL
LN

3.4.3


*
**
% CD62L+ NK cells
120
100
80
60
40
20
0
PB
AL
LN
CD2-
CD2+

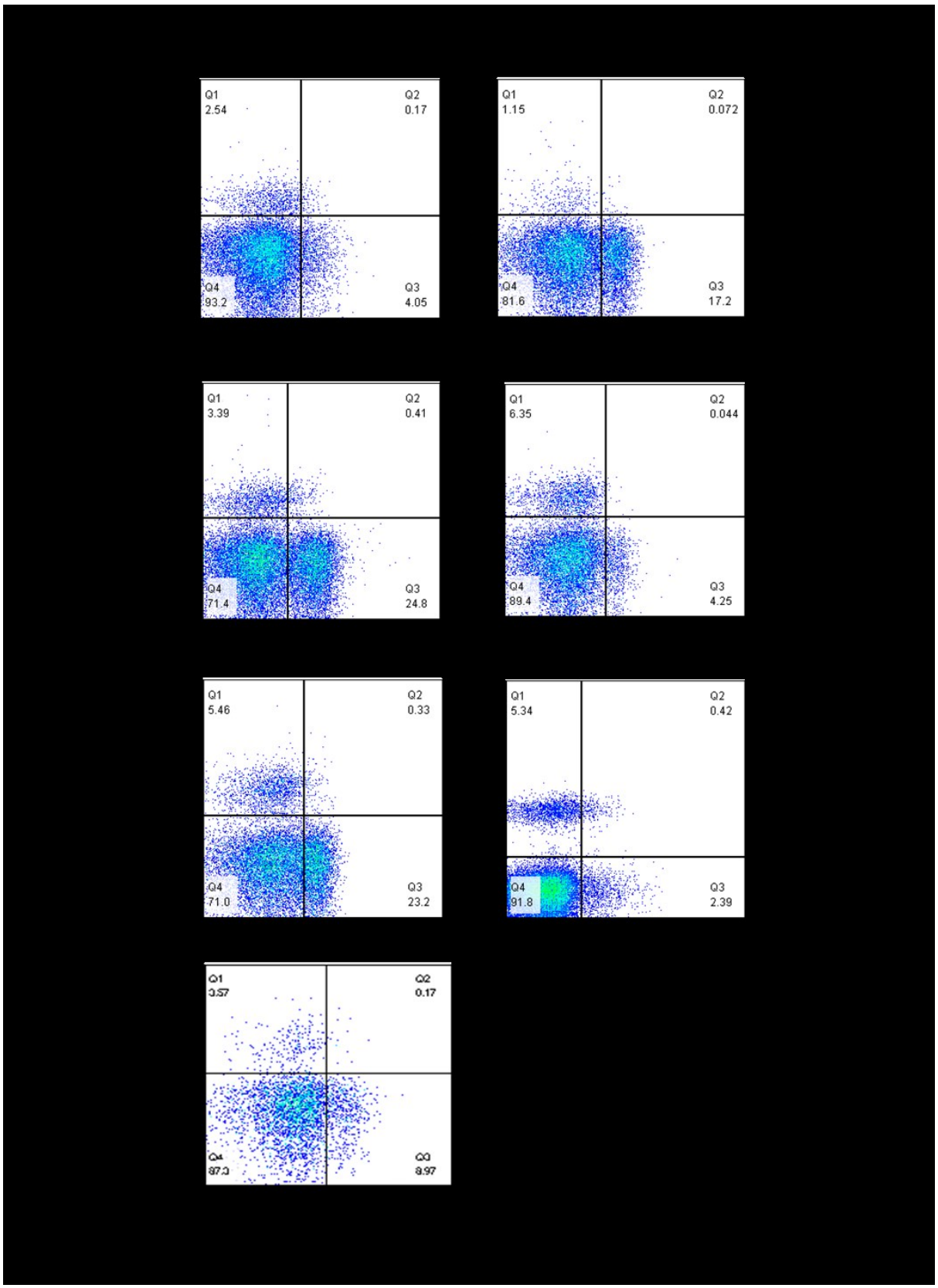

Q1
2.54
Q2
0.17
Q4
93.2
Q3
4.05
Q1
1.15
Q2
0.072
Q4
81.6
Q3
17.2
Q1
3.39
Q2
0.41
Q4
71.4
Q3
24.8
Q1
6.35
Q2
0.044
Q4
89.4
Q3
4.25
Q1
5.46
Q2
0.33
Q4
71.0
Q3
23.2
Q1
5.34
Q2
0.42
Q4
91.8
Q3
2.39
Q1
0.17
Q2
0.17
Q3
8.97

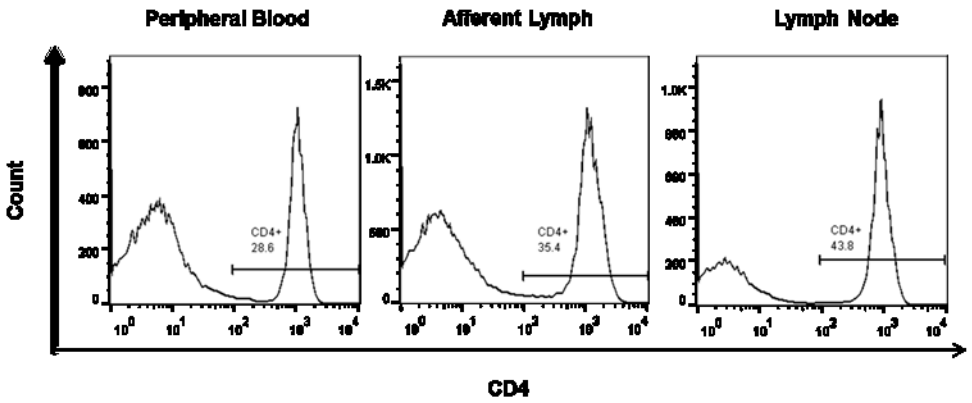
Figure 3.4 Migratory potential of NK cells present in PB, AL and LNs

Lymphocytes from PB (fresh), AL (fresh) and the LNs (fresh) of seven calves were labelled with mAbs for NKp46, CD2, CD62L and CCR7 and analysed by flow cytometry. FACS plots from one representative animal illustrate the expression of NKp46 and CD62L by PB, AL and LN derived lymphocytes (Figure 3.4.1). Quadrants were set based on FMO controls. Pooled data from seven calves indicate the percentage of NKp46+ CD62L+ NK cells present across the three compartments (Figure 3.4.2). Pooled data from seven calves illustrates the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ CD62L+ population from PB, AL or LNs (Figure 3.4.3). FACS plots illustrate the expression of CCR7 by AL derived NK cells from seven calves (Figure 3.4.4). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.05$*, $p<0.01$**.

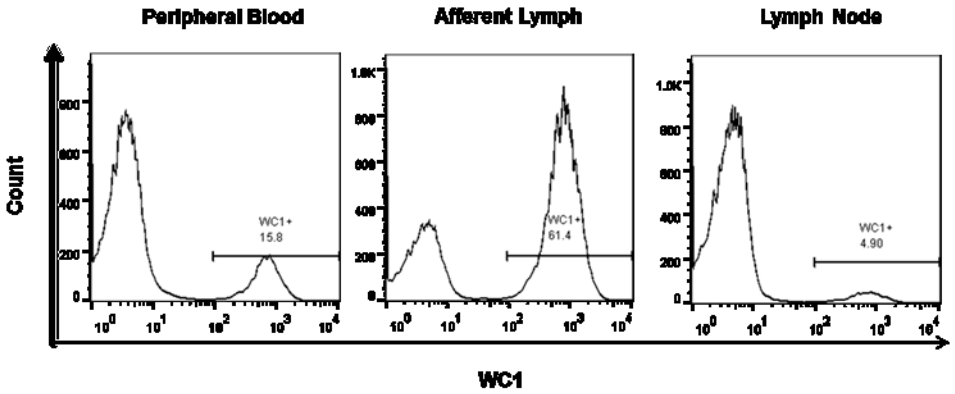
3.2.5 Percentage of CD4+ T cells and WC1+ γδ T cells present in PB, AL and LNs

To compare the frequency of NK cells present in PB, AL and LNs with the frequency of other lymphocyte populations, the percentage of CD3+CD4+ T cells and CD3+WC1+ γδ T cells present in PB, AL and the LNs was assessed. Lymphocytes were labelled with mAbs to CD3/CD4 or CD3/WC1 to define CD4+ T cells and WC1+ γδ T cells respectively and cells were analysed by flow cytometry. CD4+ T cells accounted for 19.9% (13.4 - 28.6%; SD = 5.2), 27.7% (13 - 43.8%; SD = 9.2), and 31.6% (19.4 - 52.1%; SD = 11.3) of lymphocytes present in PB, AL and LNs respectively, and were significantly increased in LNs compared with PB (p=0.043) (Figure 3.5.3). WC1+ γδ T cells represented 22.3%, 40% and 6.1% of lymphocytes found in PB, AL and LNs respectively, and were significantly decreased in LNs compared with PB (p=0.002) and AL (p=0.002). Despite the increased presence of WC1+ γδ T cells in AL, this was not significantly increased compared with PB (p=0.056) (Figure 3.5.4).

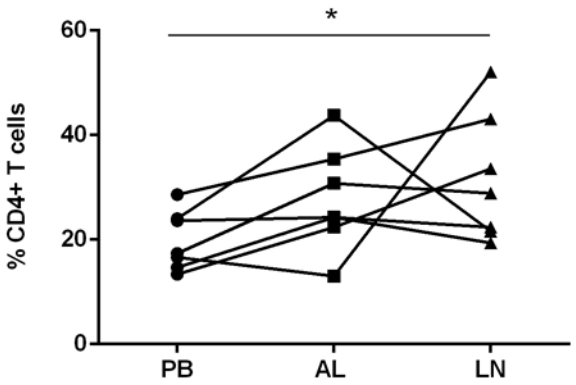
3.5.1



3.5.2



3.5.3



3.5.4

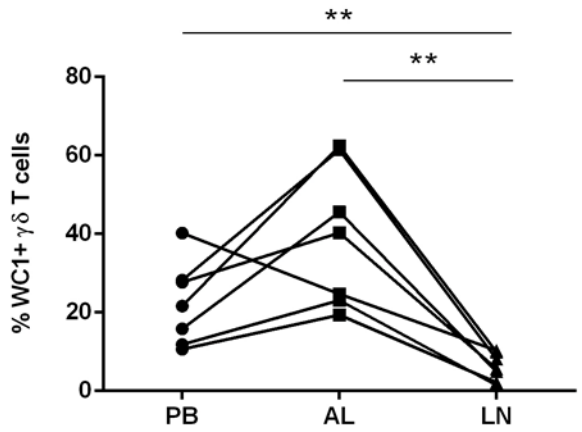


Figure 3.5 Percentage of CD4+ T cells and WC1+ γδ T cells present in PB, AL and LNs

Lymphocytes derived from PB (fresh), AL (fresh) and the LNs (fresh) of seven calves were labelled with mAbs for CD3, CD4 and WC1 and analysed by flow cytometry. FACS plots from one representative animal illustrate the expression of CD4 (Figure 3.5.1) and WC1 (Figure 3.5.2) within the total gated CD3+ population. Gates were set based on FMO controls. Pooled data from seven calves display the percentage of CD3+CD4+ T cells (Figure 3.5.3) and CD3+WC1+ γδ T cells (Figure 3.5.4) present within PB (circles), AL (squares) and LNs (triangles). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.05$*, $p<0.01$**.

3.2.6 Percentage of NK cells present in the skin

Data presented thus far provides evidence that bovine NK cells are present within skin draining afferent lymphatic vessels in steady-state conditions. However, it is not known if NK cells are resident in the skin of naïve cattle and if their presence in the skin draining afferent lymphatic vessels reflects migration from the skin or recruitment from elsewhere. Therefore, small pieces of skin were enzymatically digested and assessed for the presence of NK cells by labelling lymphocytes with mAbs to NKp46 and CD2 and analysing by flow cytometry. NKp46+ NK cells were present in the skin of three naïve calves and accounted for a mean of 2.9% (1.5 – 4.2%; SD = 1.1) of the lymphocytes present in the skin (Figure 3.6). CD2+ NK cells were the main subset of NK cells present in the skin and accounted for 83.9% (77.3 - 96.2%; SD = 8.8) of NK cells present (Figure 3.6).

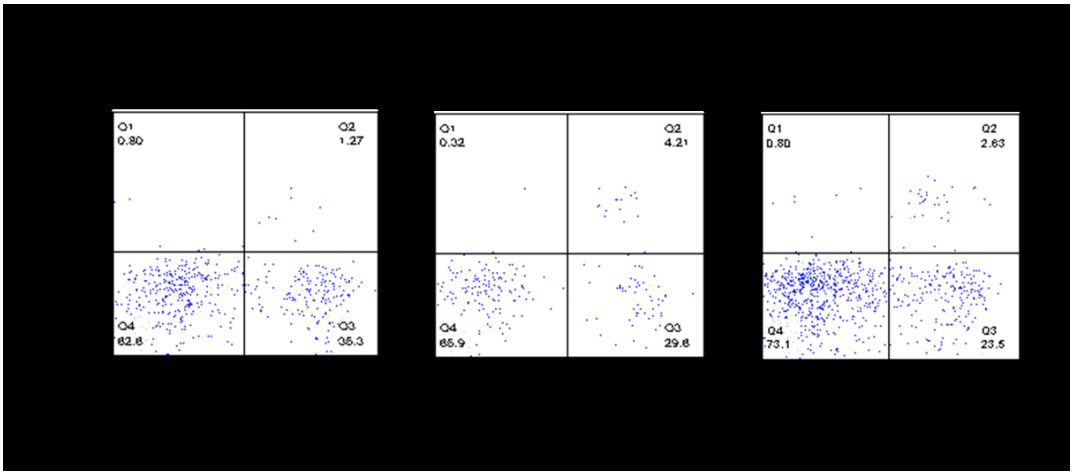


Figure 3.6 Percentage of NK cells present in the skin

Small sections of skin were removed from calves at post mortem and enzymatically digested to obtain lymphocytes. 1 x 10^6 lymphocytes (fresh) were labelled with a mAbs to NKp46 and CD2 and analysed by flow cytometry. FACS plots from three calves (303019, 403020 and 603022) illustrate the expression of NKp46 and CD2 by skin derived lymphocytes. Quadrants were set based on FMO controls.

3.2.7 Comparison between PB and EL derived lymphocytes

After establishing that NK cells were present in the skin of naïve calves and are therefore may migrate from the skin into the draining LNs via the afferent lymphatic vessels, the next question which was addressed was whether bovine NK cells were present in the efferent lymphatic vessel and therefore able to egress from the LNs in steady-state conditions. Samples of EL from five naive efferent lymphatic cannulated calves were kindly provided by Dr Bernardo Villareal-Ramos (APHA) and matched PBMCs from three of these calves were also provided. Initially to ensure the cellular composition of the PB from the efferent lymphatic cannulated calves was similar to the composition of the PB from the afferent lymphatic cannulated calves (as illustrated in Figures 3.1 - 3.5), the frequency and phenotype of NK cells derived from matched PB and EL from three calves was investigated. The percentage of NKp46+ CD3- NK cells and the distribution of CD2+ and CD2- subsets within PB and EL were assessed by labelling lymphocytes with mAbs to NKp46, CD3 and CD2 and analysing by flow cytometry. NK cells accounted for 0.68% (0.48 - 0.94%; SD = 0.2) of lymphocytes present in EL, compared with 7.6% (3.23 - 15.3%; SD = 5.5) found in PB (Figure 3.7.1). Within PB, 80.8% (74.3 - 93.2%; SD = 8.7) of NK cells present were CD2+, whereas 66.5% (57.5 - 79.4%; SD = 9.3) of the NK cells found in EL were CD2-. The percentage of CD2- NK cells was significantly increased in EL ($p=0.014$) compared with PB (Figure 3.7.2).

The activation status of PB and EL derived NK cells were measured by assessing the expression of CD25. Lymphocytes were labelled with mAbs to NKp46, CD2 and CD25 and analysed by flow cytometry. 15.7% (8.4 - 22.3%; SD = 5.7) of NK cells from PB were CD25+, compared to a significantly ($p=0.009$) higher proportion of CD25+ NK cells in EL (73.7% (72.1 - 76.6%; SD = 2.1)) (Figure 3.7.3). The percentage of CD2- NK cells positive for CD25 was significantly increased in the EL compared with PB ($p=0.048$) (Figure 3.7.4).

The frequency of bovine CD4+ T cells and γδ T cells within the efferent lymphatic vessel of the LNs draining the skin has been previously documented (Vrieling et al., 2012). Therefore, to ensure the samples of EL obtained from APHA were similar to the published literature, the percentage of CD3+CD4+ T cells and CD3+WC1+ γδ T

cells present in PB and EL were compared. Lymphocytes were labelled with mAbs to CD3/CD4 or CD3/WC1 and analysed by flow cytometry. Within PB, CD4+ T cells accounted for 37.6% (27.2 - 53.6%; SD = 11.5) of lymphocytes present, compared with 14.1% (10.7 - 16.5%; SD = 2.5) found in EL (Figure 3.7.5). No significant differences between the percentages of CD4+ T cells present in PB and EL were noted. WC1+ γδ T cells represented 13% (10.4 - 14.5%; SD = 1.8) and 45% (35.4 - 50.5%; SD = 6.8) of lymphocytes within PB and EL respectively and WC1+ γδ T cells were significantly increased in EL compared with PB ($p=0.023$) (Figure 3.7.6).

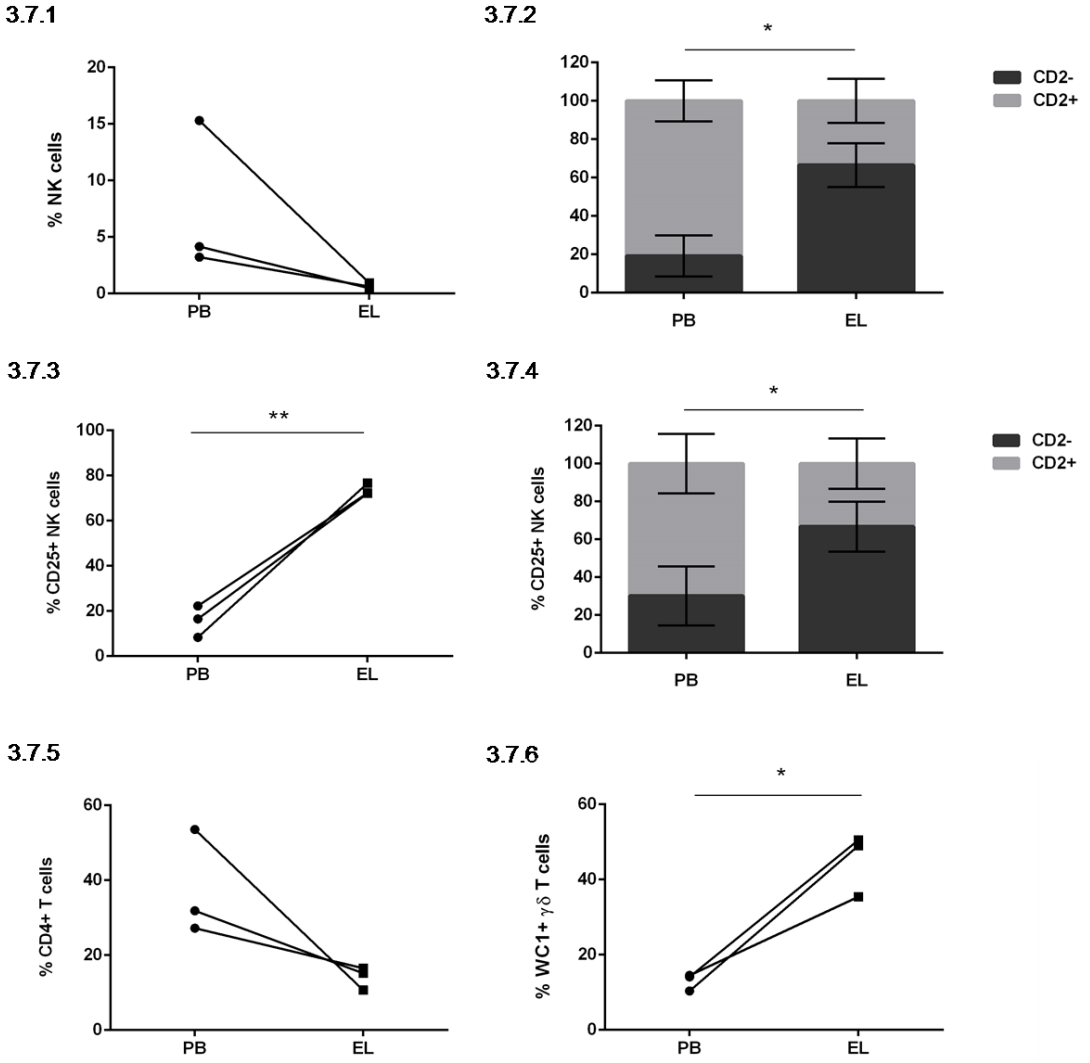


Figure 3.7 Comparison between PB and EL derived lymphocytes

Lymphocytes derived from PB (frozen) and EL (frozen) of three calves were labelled with mAbs to NKp46, CD3, CD2, CD25, CD3, CD4 and WC1 and analysed by flow cytometry. Gates were set based on FMO controls. Pooled data from three calves show the percentage of: NKp46+ CD3- NK cells (Figure 3.7.1); CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population (Figure 3.7.2); CD25+ NK cells within the total gated NKp46+ NK cell population (Figure 3.7.3); CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ CD25+ NK cell population (Figure 3.7.4); CD3+CD4+ T cells (Figure 3.7.5) and CD3+WC1+ γδ T cells (Figure 3.7.6) present within PB (circles) and EL (squares). Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.05$*, $p<0.01$**.

3.2.8 Percentage of NK cells present in PB, AL, LNs and EL

After confirming the samples of PB from APHA (Figures 3.7.1 - 3.7.4) were of a similar cellular composition to the PB from the afferent lymphatic cannulated calves and the samples of EL were equivalent to that of the published literature (Figure 3.7.5 and Figure 3.7.6), the frequency and phenotype of EL derived NK cells were directly compared with NK cells from PB, AL and LNs. NK cells represented 1% (0.48 - 1.58%; SD = 0.4) of lymphocytes within EL and NK cell frequency was significantly lower in EL compared with PB ($p=0.020$), AL ($p=0.003$) and LNs ($p=0.006$) (Figure 3.8.1). Similar to NK cells present in AL and LNs, 71.7% (66.8 - 76.6%; SD = 3.4) of EL derived NK cells were CD2- and CD2- NK cells were present at a significantly higher percentage in EL compared with PB ($p<0.001$) (Figure 3.8.2).

3.2.9 Activation of NK cells present in PB, AL, LNs and EL

The expression of CD25 by EL derived NK cells was compared with those from PB, AL and LNs. 71.2% (66.8 - 76.6%; SD = 3.4) of NK cells from EL were CD25+ (Figure 3.9.1) and similar to those present in AL and the LNs, the percentage of CD25+ NK cells ($p<0.001$) and the MFI of CD25+ NK cells ($p=0.043$) was significantly increased in EL compared with PB (Figure 3.9.1 and Figure 3.9.2 respectively). Furthermore, when assessing the subset of NK cells responsible for this enhanced activation of NK cells in the EL, 63.3% (45.1 - 77.3%; SD = 12.5) of CD25+ NK cells were CD2-, however this was not significantly ($p=0.069$) different to the percentage of CD2- CD25+ NK cells present in PB, AL or LNs (Figure 3.9.3).

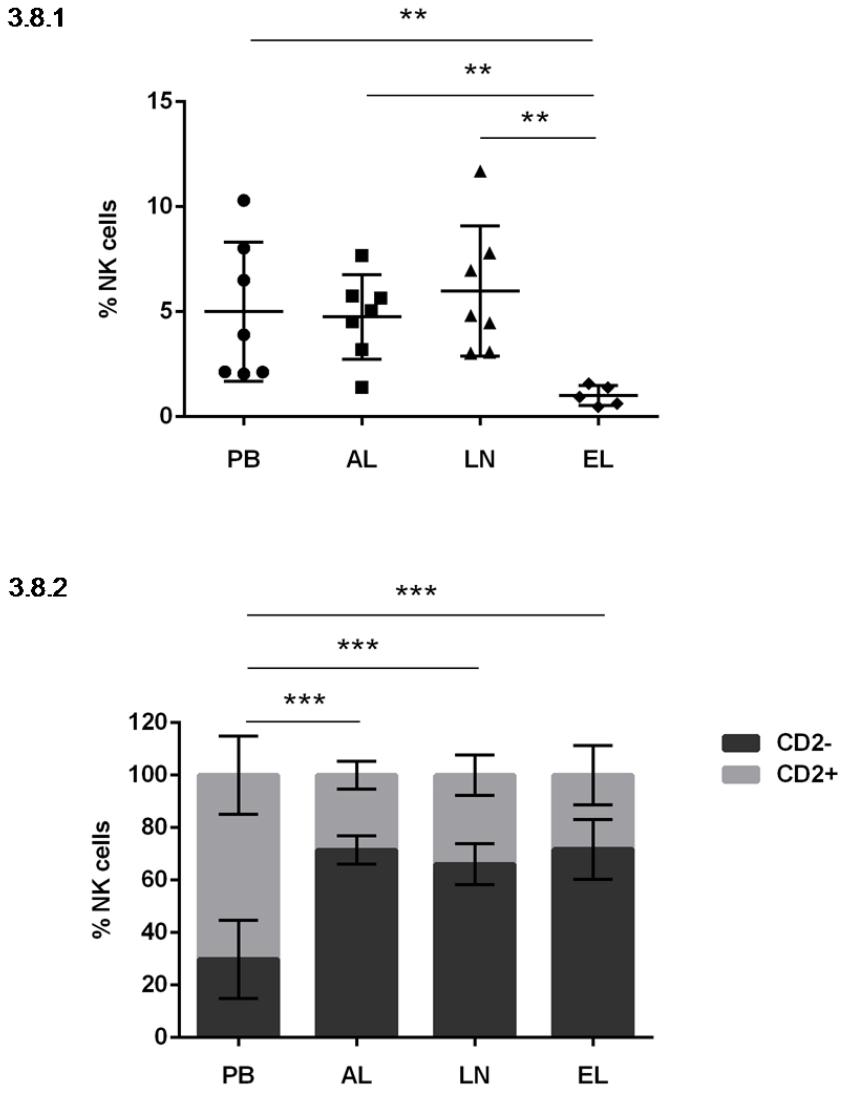
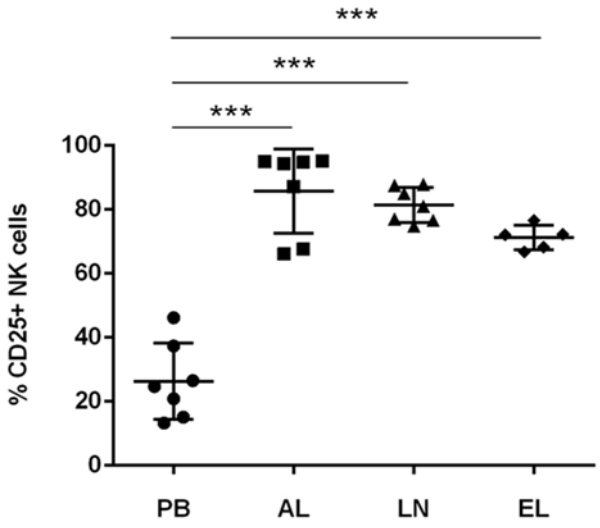


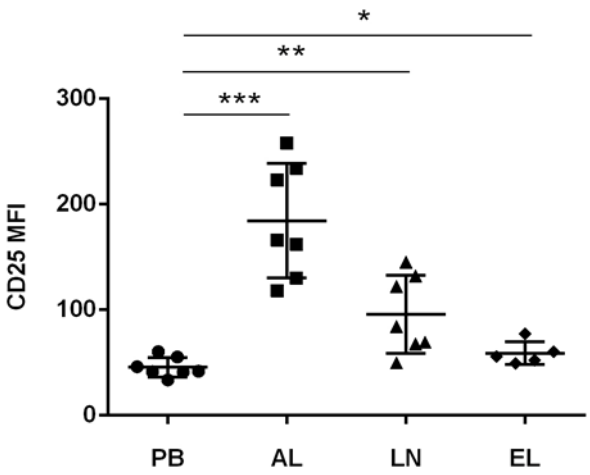
Figure 3.8 Percentage of NK cells present in PB, AL, LNs and EL

Lymphocytes derived from PB, AL, LNs (fresh) and EL (frozen) were labelled with mAbs for NKp46, CD3 and CD2 and analysed by flow cytometry. Pooled data from seven calves for PB, AL and LNs (Figure 3.1.2), and five calves for EL, show the average percentage of NKp46+ CD3- NK cells ± SD present within PB (circles), AL (squares), LNs (triangles) and EL (diamonds) (Figure 3.8.1). Pooled data from seven (PB, AL and LNs) and five (EL) calves illustrates the average percentage of NKp46+ CD2+ (lighter bars) and NKp46+ CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population from PB, AL, LN (Figure 3.2.2), and five calves for EL (Figure 3.8.2). Data were normally distributed ($p>0.05$) and significance was assessed using two sample and paired t-tests. $p<0.01$**, $p<0.001$***.

3.9.1


***
***
***
% CD25+ NK cells
100
80
60
40
20
0
PB
AL
LN
EL

3.9.2


*
**
***
CD25 MFI
300
200
100
0
PB
AL
LN
EL

3.9.3

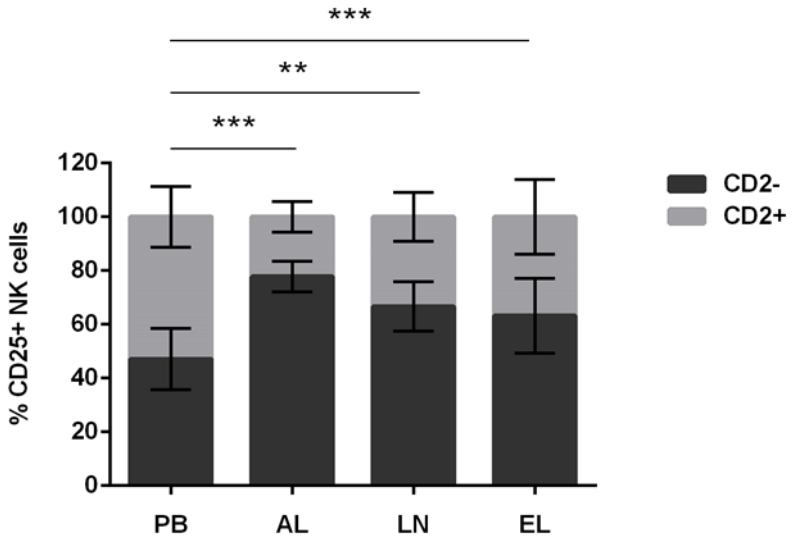

***
**
***
% CD25+ NK cells
120
100
80
60
40
20
0
PB
AL
LN
EL
CD2-
CD2+

Figure 3.9 Activation of NK cells present in PB, AL, LNs and EL

Lymphocytes derived from PB, AL, LNs (fresh) and EL (frozen) were labelled with mAbs to NKp46, CD2 and CD25 and analysed by flow cytometry. Pooled data from seven calves for PB, AL and LNs (Figure 3.3.2), and five calves for EL, illustrate the average percentage of CD25+ NK cells ± SD within the total NKp46+ population from PB (circles), AL (squares), LNs (triangles) and EL (diamonds) (Figure 3.9.1). Pooled data from seven calves for PB, AL and LNs (Figure 3.3.3), and five calves for EL, indicate the average MFI of CD25+ NK cells ± SD within the total NKp46+ population from PB (circles), AL (squares), LNs (triangles) and EL (diamonds) (Figure 3.9.2). Pooled data from seven (PB, AL and LNs) and five (EL) calves indicate the average percentage of NKp46+ CD2+ (lighter bars) and NKp46+ CD2- (darker bars) NK cells ± SD within the total gated NKp46+ CD25+ NK cell population from PB, AL, LN (Figure 3.3.4) and EL (Figure 3.9.3). Data were normally distributed ($p>0.05$) and significance was assessed using two sample and paired t-tests. $p<0.05$*, $p<0.01$**, $p<0.001$***.

3.2.10 Percentage of CD4+ T cells and WC1+ γδ T cells in PB, AL, LNs and EL

The percentage of CD4+ and WC1+ γδ T cells across PB, AL, LNs and EL (frozen) were compared by labelling lymphocytes with mAbs to CD3/CD4 and CD3/WC1. CD4+ T cells accounted for 31.2% (18 - 53.6%; SD = 12.1) of lymphocytes present within EL, which was not significantly different to the percentage of CD4+ T cells found in PB, AL or LNs (Figure 3.10.1). WC1+ γδ T cells represented 46.9% (35.4 - 52.8%; SD = 6.1) of lymphocytes within EL and were significantly increased in EL compared with PB ($p=0.001$) and LNs ($p<0.001$) (Figure 3.10.2).

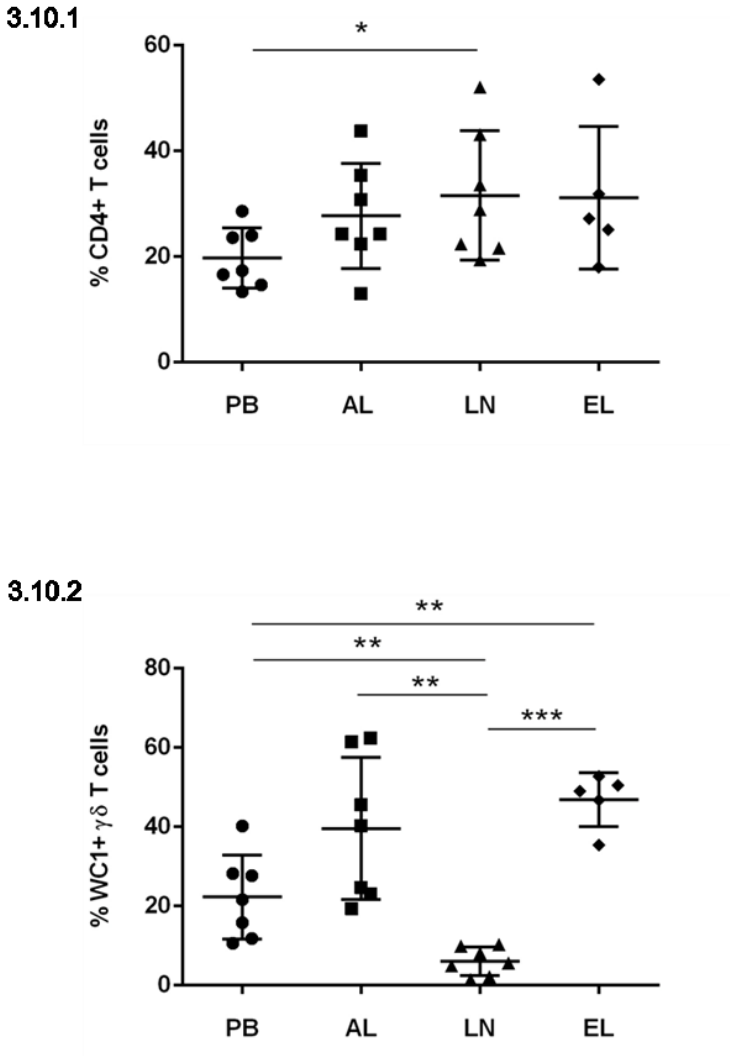


Figure 3.10 Percentage of CD4+ T cells and WC1+ γδ T cells in PB, AL, LNs and EL

Lymphocytes derived from PB, AL, LNs (fresh) and EL (frozen) were labelled with mAbs for CD3/CD4 and CD3/WC1 and analysed by flow cytometry. Pooled data from seven calves for PB, AL and LNs (Figure 3.5.3), and five calves for EL, illustrate the average percentage of CD3+ CD4+ T cells ± SD present within PB (circles), AL (squares), LNs (triangles) and EL (diamonds) (Figure 3.10.1). Pooled data from seven (PB, AL and LNs) and five (EL) calves indicate the average percentage of WC1+ γδ T cells ± SD from PB, AL, LN (Figure 3.5.4) and EL (Figure 3.10.2). Data were normally distributed ($p>0.05$) and significance was assessed using two sample t-tests. $p<0.05$*, $p<0.01$**, $p<0.001$***.

3.3 Discussion

The development of a mAb to bovine NKp46 has revolutionised bovine NK cell research (Storset et al., 2004). NK cells are widely distributed in lymphoid and non-lymphoid tissues, but very little is known about how NK cells migrate to their anatomical locations and their recirculation between blood and tissues. It was demonstrated recently that bovine NK cells are present within the afferent lymphatic vessels draining the skin of cattle in the steady-state, suggesting that they can traffic from tissues into LNs via the lymphatics. In that study, the frequency, phenotype and function of NK cells from PB and AL of different calves were compared (Lund et al., 2013). As the samples of PB and AL were not from the same animal, it is more difficult to draw firm conclusions about the differences between these two compartments. Data presented herein aimed to extend the findings by Lund *et al* by analysing the frequency and phenotype of NK cells and NK cell subsets in parallel from the PB, AL and LNs of the same animal, achieved by utilising the bovine pseudo-afferent lymphatic cannulation model (Hope et al., 2006).

NKp46+ CD3- NK cells were present in AL draining the skin of naïve calves, at a slightly reduced frequency compared with those found in PB (Figure 3.1.3). This illustrates that bovine NK cells can traffic from tissues such as the skin to the draining LNs via afferent lymphatic vessels. In line with these findings, NK cells were found to be more abundant in PB than AL in a study involving non-matched samples of PB and AL (Lund et al., 2013). Furthermore in humans, NK cells were found to be present at significantly lower levels in the AL compared with PB (Yawalkar et al., 2000, Hunger et al., 1999) and recently, NK cells were shown to be present in seroma fluid (an accumulation of AL), representing greater than 2% of lymphocytes (Carrega et al., 2014). In this study, bovine LNs contained the highest percentage of NK cells across the three compartments examined (Figure 3.1.1). This abundance of NK cells within bovine LNs is in line with published findings (Boysen et al., 2008) and is also similar to the increased number of NK cells present in human LNs in steady-state conditions (Fehniger et al., 2003). This is in contrast to mouse LNs where NK cells account for approximately 0.5% of lymphocytes present (Gregoire et al., 2007).

It is well established in the literature that within the PB of cattle, the majority of NK cells are CD2+, whereas CD2- NK cells are the predominant subset found within the LNs (Boysen et al., 2006). Data presented in Figure 3.2.2 support this paradigm, whereby the percentage of CD2- NK cells present within the PB was low, but was significantly increased in AL and LNs, reflecting preferential recruitment of the CD2- subset of NK cells from the tissues to the draining LNs in steady-state conditions. In studies of human seroma fluid, $CD56^{bright}$ NK cells were the main subset of NK cells present (Carrega et al., 2014) and parallels have been drawn between this subset of human NK cells and the CD2- subset of bovine NK cells (as illustrated in Figure 1.2).

AL and LNs draining the skin of cattle in steady-state conditions contained highly activated NK cells compared with those found within PB (Figure 3.3.2 and Figure 3.3.3). CD25 is an activation marker expressed by activated T and B cells. The observed activation of NK cells in AL and LNs may be caused by interactions with DCs or increased responsiveness to T cell derived IL-2, together resulting in the augmented expression of CD25 by NK cells. CD2- NK cells were more activated in AL and LNs than CD2+ NK cells (Figure 3.3.4). In line with this finding, bovine CD2- NK cells are preferably activated when stimulated with IL-2 compared with CD2+ NK cells (Boysen et al., 2006). In addition to CD25, the expression of a second activation marker CD44, was noted to be significantly increased by AL NK cells which highlights an overall activated phenotype within AL (Lund et al., 2013). Furthermore, CD4+ and CD8+ T cells expressing markers of activation including CD25, CD69 and MHC class II were present in AL of humans in steady-state conditions (Yawalkar et al., 2000). Taken together, this indicates that events occurring as a result of egress from the skin, results in the arrival of tissue activated NK cells in the AL and LNs.

The migratory potential of NK cells derived from PB, AL and LNs was characterised by assessing CD62L and CCR7 expression. Through interactions with HEVs, NK cell expression of CD62L allows homing to LNs (Chen et al., 2005). There were no significant differences in the percentage of CD62L+ NK cells across the three compartments however a slightly lower percentage of CD62L+ NK cells were present in the LNs which correlates with the down-regulation of CD62L in IL-2 rich areas (Boysen et al., 2008) (Figure 3.4.2). Despite there being no significant changes in the

expression of CD62L by NK cells across PB, AL and LNs, differences in the percentage of CD2+ and CD2- NK cells expressing CD62L were evident with an increased percentage of CD2- CD62L+ NK cells in the AL and LNs, compared with PB (Figure 3.4.3). This elevated expression of CD62L by CD2- NK cells in the AL and LNs correlates with their prevalence within these two compartments. Bovine NK cells transcribe the LN homing chemokine receptor, CCR7 (Siddiqui and Hope, 2012), however surface CCR7 expression could not be detected by PB or LN derived NK cells (data not shown). Using an anti-human CCR7 antibody cross-reactive to bovine cells, the expression of CCR7 by AL NK cells was very variable across the seven calves examined (FACS plots from seven calves are illustrated in Figure 3.4.4) and expression was not detected by AL NK cells in previously published work (Lund et al., 2013). In humans, CCR7 is expressed by $CD56^{bright}$ NK cells but is absent from $CD56^{dim}$ NK cells which coincides with the dominance of the $CD56^{bright}$ subset of NK cells in AL and LNs (Maghazachi, 2010). Bovine γδ T cells traffic to LNs in a CCR7-independent manner (Vrieling et al., 2012) and therefore bovine NK cells may also move to the LNs in a similar CCR7-independent manner. Further investigation is required to decipher key molecules utilised to permit NK cell migration in cattle.

Across all three compartments, CD4+ T cells were present at the highest frequency in LNs (Figure 3.5.3). In contrast, LNs contained a low frequency of WC1+ γδ T cells compared with PB and AL (Figure 3.5.4), which has been documented previously and reflects minimal interactions of γδ T cells within the LNs (Vrieling et al., 2012). This theory is supported in sheep where ovine γδ T cells migrate through LNs with greater efficiency than αβ T cells and are localised to interfollicular traffic areas of the LNs (Young et al., 2000). Unlike γδ T cells, bovine NK cells are present at relatively high frequencies in the LNs (Figure 3.1.2), where they are localised to the paracortex and the medulla (Boysen et al., 2008) and can undergo priming as a result of interactions with DCs (Lucas et al., 2007).

NK cells are resident within bovine skin and the majority were of the CD2+ NK cell subset (Figure 3.6). In comparison to CD2- NK cells, which transcribe lymphoid homing chemokine receptors, CD2+ NK cells are more likely to be non-migratory due to the absence of appropriate receptors that govern homing to secondary lymphoid

organs and this may reflect their abundance in the skin (Siddiqui and Hope, 2012). The skin material analysed for the presence of NK cells was not from the calves used for pseudo-afferent lymphatic cannulation, therefore quantifying the frequency and phenotype of skin NK cells from the afferent lymphatic cannulated calves would allow direct comparison of skin NK cells with PB, AL and LN derived NK cells in the same animal. In addition, histology of skin sections would confirm the presence of NK cells in bovine skin and their precise location. Nevertheless, this preliminary experiment provides some evidence that NK cells are found in the skin of calves and therefore suggests that NK cells can migrate from the skin, through afferent lymphatic vessels and into the LNs. In humans, $CD56^{bright}$ CD16- NK cells are present in the dermis of healthy skin and were also found in skin from patients with atopic eczema/dermatitis, where they were in close contact with CD1a+ DCs (Buentke et al., 2002, Ebert et al., 2006).

After establishing that NK cells can migrate from the skin via afferent lymphatic vessels and into the draining LNs in steady-state conditions, samples of EL were analysed for the presence or absence of NK cells to understand the possible re-circulatory nature of NK cells. Initial experiments focused on comparing matched PB and EL from three calves. It was confirmed that PB derived NK cells from efferent lymphatic cannulated calves were of a similar frequency (Figure 3.7.1) and phenotype (Figure 3.7.3) to PB derived NK cells from afferent lymphatic cannulated calves and that the cellular composition (CD4+ T cells and WC1+ γδ T cells) of the EL samples were comparable to the published literature (Figure 3.7.5 and Figure 3.7.6) (Vrieling et al., 2012). Therefore, samples of EL from five naive efferent lymphatic cannulated calves were directly compared to PB, AL and LNs of the seven afferent lymphatic cannulated calves, allowing comparison across the four compartments.

NK cells were present in EL, at lower levels than those found in PB, AL and LNs, providing evidence that a proportion of NK cells can exit the LNs via the efferent lymphatic vessel and therefore may re-circulate in steady-state conditions (Figure 3.8.1). Similar to NK cells entering the LNs via the afferent lymphatic vessel and those within LNs, EL derived NK cells were predominantly CD2- (Figure 3.8.2), illustrating the CD2- NK cells may be the preferred subset to egress from the nodes and return to

circulation. To date, one study has shown that NK cells are present within human EL of humans which were predominantly of the $CD56^{bright}$ subset (Romagnani et al., 2007). The presence of NK cells in the efferent lymphatic vessels of other species has not been defined. Thus, it appears that in cattle and in humans, CD2- NK cells and $CD56^{bright}$ NK cells, which are the principal subsets of NK cells within the LNs, are also the main subsets egressing from the LNs to return to circulation. T and B cells utilise sphingosine-phosphate-receptor 1 (S1P1) to egress from peripheral lymphoid organs (Matloubian et al., 2004, Pappu et al., 2007), however down-regulation of S1P1 through administration of FTY720, did not deplete NK cells from the circulation of mice or humans (Vaessen et al., 2006, Walzer et al., 2007b). Consequently, it was demonstrated that NK cells express sphingosine-phosphate-receptor 5 (S1P5) which permits NK cell exit from the bone marrow and LNs in mice (Mayol et al., 2011, Jenne et al., 2009). It would be valuable to assess the expression of S1P receptors by bovine NK cells to understand the molecules required to exit peripheral lymphoid organs.

In conjunction with AL and LN derived NK cells, NK cells within EL were activated (Figure 3.9.1 and Figure 3.9.2) which was attributed to the CD2- subset of NK cells (Figure 3.9.3). Similarly, human EL derived NK cells display an activated phenotype reflected by their increased expression of CD16 and KIR in comparison with NK cells in the LNs (Romagnani et al., 2007). Therefore, it is conceivable that tissue activated CD2- CD25+ NK cells arriving in the LNs also egress from the LNs via the efferent lymphatic vessel and circulate in a primed state in steady-state conditions.

In summary, data presented in this Chapter and illustrated in Figure 3.11, has demonstrated that bovine NK cells may be present in the skin of cattle in steady-state conditions and that the CD2- subset of NK cells are preferentially recruited from the tissues, through their expression of CD62L, into draining LNs via afferent lymphatic vessels in a highly activated state. Similarly, NK cells within bovine LNs are predominantly CD2- NK cells with a high expression of CD25 and CD62L, suggesting that NK cells could be primed in both AL and LNs. Furthermore, the presence of CD2- CD25+ NK cells within EL provides evidence that a proportion of NK cells do not terminally reside in the LNs and can egress from the nodes to return to circulation where they are predominantly CD2+ $CD25^{lo}$. To conclude, experimental findings in

this Chapter provide evidence that the frequency and phenotype of NK cells in cattle is reflective of their anatomical location, therefore proving the hypothesis stated at the beginning of this Chapter. The effect of BCG vaccination on PB, AL, LN and EL derived NK cells will be described in Chapter 4.

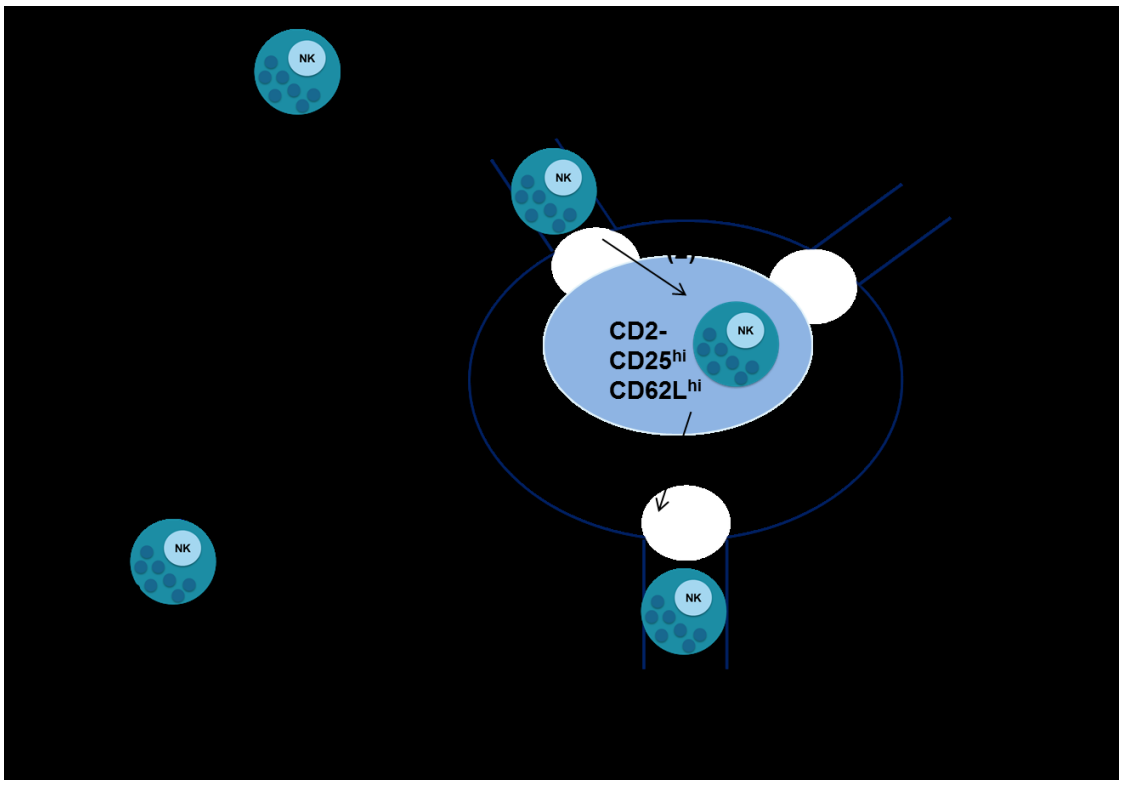


Figure 3.11 A model to illustrate bovine NK cell re-circulation in steady-state conditions

Bovine NK cells can migrate from the skin, through afferent lymphatic vessels (1) and into the skin draining LNs (2). NK cells can then egress from the LNs via the efferent lymphatic vessel (3) and return to circulation (4). NK cells derived from the afferent lymphatic vessel, LNs and efferent lymphatic vessel are predominantly CD2- NK cells which have a high expression of CD25. Conversely, NK cells present in the blood are CD2+ NK cells with a low expression of CD25.

Abstract

Vaccination of neonatal calves with BCG provides significant protection against infection with *M. bovis* therefore NK cell responses in the blood, afferent lymph, lymph node and efferent lymph draining the site of BCG vaccination were assessed. The percentage and receptor repertoire of NK cells were altered in the blood following BCG vaccination of neonatal calves. No evidence of NK cell recruitment, or changes in phenotype, were noted in the lymph nodes draining the site of BCG vaccination in both vaccinated and revaccinated calves. Preliminary experiments using pseudo-afferent or efferent lymphatic cannulated calves vaccinated with BCG suggested changes in NK cell responses in the lymph following vaccination. Together, this data suggests a potential role of NK cells during BCG vaccination of neonatal calves.

4. The effect of BCG vaccination on peripheral blood, afferent lymph, lymph node and efferent lymph derived NK cells

4.1 Introduction

Vaccination is thought to be the cornerstone of future bTB disease control, alongside improved DIVA diagnostic tests which differentiate infected from vaccinated animals. However, currently there are no vaccines licensed for use in cattle. Despite over a century of research, BCG a live attenuated strain of *M. bovis*, remains the only vaccine available for use in humans against TB. This vaccine is particularly effective when delivered to infants to induce protection against tuberculous meningitis and miliary TB (Trunz et al., 2006, Bonifachich et al., 2006). Similarly, a number of studies have demonstrated that experimental vaccination of neonatal calves with BCG provides significant protection against *M. bovis* infection (Buddle et al., 1995a, Hope et al., 2005, Hope et al., 2011). Similar to humans, neonatal vaccination strategies are effective in calves as they are born with a competent immune system and therefore respond well to vaccination at a young age. Furthermore, vaccination of neonatal calves overcomes the negative effects associated with pre-exposure to environmental mycobacteria prior to vaccination, which can result in stimulation of an inappropriate immune response (Brandt et al., 2002). Nevertheless, the use of BCG in the field is prohibited by EU legislation due to non-specific sensitisation to the tuberculin skin test. Despite this, BCG remains the most effective vaccine available for use and continued development of DIVA tests may allow it to be deployed in the UK for bTB control.

BCG vaccination of infants induces activation of innate effector cells such as NK cells and γδ T cells (Zufferey et al., 2013), and since young calves, particularly those aged between 8-120 days old have increased circulating numbers of NK cells (Graham et al., 2009, Kulberg et al., 2004, Elhmouzi-Younes et al., 2009), it was hypothesised that NK cells may play a role in the enhanced efficacy of BCG in neonatal calves. Alongside the increased frequency, NK cells derived from neonatal calves also exhibit increased activity, reflected by a higher expression of perforin compared with NK cells from older animals and enhanced cytotoxicity after culture with IL-15 (Elhmouzi-

Younes et al., 2009). It is proposed that the high numbers of innate effector cells (NK cells and γδ T cells expressing the WC1 receptor) present in young calves has evolved to compensate for the immature state of the adaptive immune system. The increased number of NK cells and WC1+ γδ T cells in young animals may be relevant to targeted vaccination strategies and induction of effective immune responses in neonatal calves. Similar to young calves, infants have elevated levels of NK cells which also decline with age (Erkeller-Yuksel et al., 1992, Sundstrom et al., 2007); therefore research focusing on the role of NK cells during mycobacterial infection or vaccination in neonatal calves may be applicable to studies in humans.

There is considerable evidence to demonstrate that NK cells are responsive to mycobacteria *in vivo* and therefore to suggest that NK cells play a role in anti-mycobacterial immunity. The role of NK cells during *M. tb* infection was recently reviewed by Esin and Batoni (Esin and Batoni, 2015). Following infection of mice with *M. tb* or BCG, increased numbers of activated NK cells are recruited to the lungs where they secrete IFN-γ (Junqueira-Kipnis et al., 2003). Similarly, during studies of active *M. tb* infection in humans, NK cells infiltrate the lungs where they are localised to granulomas (Portevin et al., 2012). Depletion of murine NK cells at the time of BCG vaccination results in increased immunosuppressive regulatory T cells, increased bacillary burden and reduced T cell responses after challenge with *M. tb* (Dhiman et al., 2012). However, the role of NK cells during BCG vaccination or *M. bovis* infection in cattle has not yet been defined.

Even though BCG has been shown to be effective in neonatal calves (Buddle et al., 1995b, Hope et al., 2005, Hope et al., 2011), similar to humans, the efficacy of BCG in calves is variable across studies (reviewed in (Waters et al., 2012)). Ideally a better vaccine which induces sterilising immunity whilst avoiding sensitisation to the tuberculin skin test would be developed. In order to do this, the immune response following BCG vaccination and the way in which BCG induces protection in neonates must be deciphered. This may pinpoint mechanisms or cellular targets for enhanced vaccination strategies. **It was hypothesised that vaccination of neonatal calves with BCG would alter NK cell properties across PB, AL, LNs and EL**, therefore indicating a role for bovine NK cells during BCG vaccination of neonatal calves and

potential mechanisms of action. Data presented in Chapter 3 highlighted significant differences in the percentage, subset distribution and phenotype of NK cells across PB, AL, LNs and EL in steady-state conditions. The studies presented herein aim to extend these findings by assessing the effect of subcutaneous BCG vaccination on the frequency, phenotype, function and receptor repertoire of PB derived NK cells in neonatal calves. Furthermore, to assess recruitment of NK cells *in vivo* following BCG vaccination, the frequency and phenotype of NK cells within the skin-draining afferent lymphatic vessels, PSLNs and efferent lymphatic vessel were assessed.

4.2 Results

4.2.1 Production of antigen-specific IFN-γ following BCG vaccination

Six week old neonatal calves were vaccinated subcutaneously with 0.5ml BCG Danish SSI, administered to the left shoulder. Age-matched, control calves were left unvaccinated. To determine if calves were responding to BCG vaccination, whole blood was sampled one week prior to BCG vaccination, on the day of vaccination (week 0) and then at two-weekly intervals following vaccination for up to 12 weeks. Whole blood was stimulated with PPD-b for 18 hours and the production of antigen-specific IFN-γ in the plasma supernatant was assessed by ELISA (section 2.15). Following BCG vaccination, PBMCs from BCG-vaccinated calves produced significantly elevated levels of antigen-specific IFN-γ at 4 weeks ($p=0.025$), 6 weeks ($p=0.02$) and 8 weeks ($p=0.023$) post-vaccination compared with week 0. Non-vaccinated calves produced low levels of IFN-γ (Figure 4.1).

4.2.2 Percentage of NK cells and subsets present in the blood following BCG vaccination

To define the effect of BCG vaccination on NK cell frequency and the distribution of CD2+ and CD2- subsets within the blood, the percentage of NK cells and the percentage of the two subsets was monitored by labelling lymphocytes with mAbs to NKp46 and CD2. Cells were analysed by flow cytometry. Following BCG vaccination, the percentage of NK cells increased significantly on day 1 post-vaccination, compared with pre-vaccination (Day 0) levels ($p<0.001$). No other significant alterations were noted post-BCG vaccination, or throughout the experiment in non-vaccinated calves (Figure 4.2). CD2+ NK cells are the principal subset of NK cells present in the blood (as illustrated in Figure 3.2.2), however no significant effect of BCG on the proportions of CD2+ and CD2- subsets of NK cells were evident throughout the vaccination experiment (data not shown).

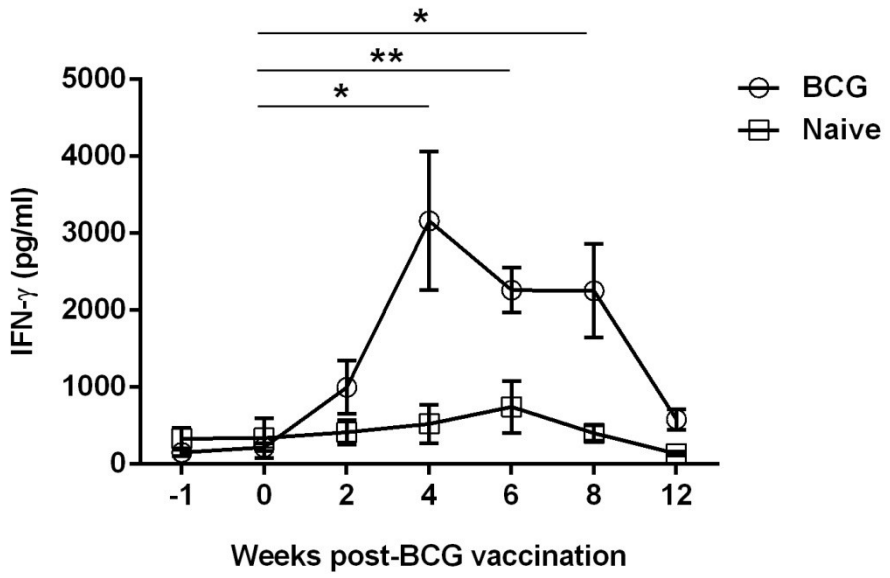


Figure 4.1 Production of antigen-specific IFN-γ following BCG vaccination

Whole blood from BCG-vaccinated and non-vaccinated calves was stimulated for 18 hours with PPD-b one week prior to vaccination, the day of vaccination and then at two weekly intervals following BCG vaccination for 12 weeks. Plasma supernatants were retrieved and the presence of IFN-γ (pg/ml) was determined by ELISA (section 2.15). Pooled data from 12 BCG vaccinated (circles) and 9 naïve (squares) calves ± SD illustrates the production of antigen-specific IFN-γ (pg/ml) after stimulation of whole blood with PPD-b at various time points post-BCG vaccination. Data were normally distributed ($p>0.05$), and significance was assessed using GLM and paired t tests. $p<0.05$*, $p<0.01$**.

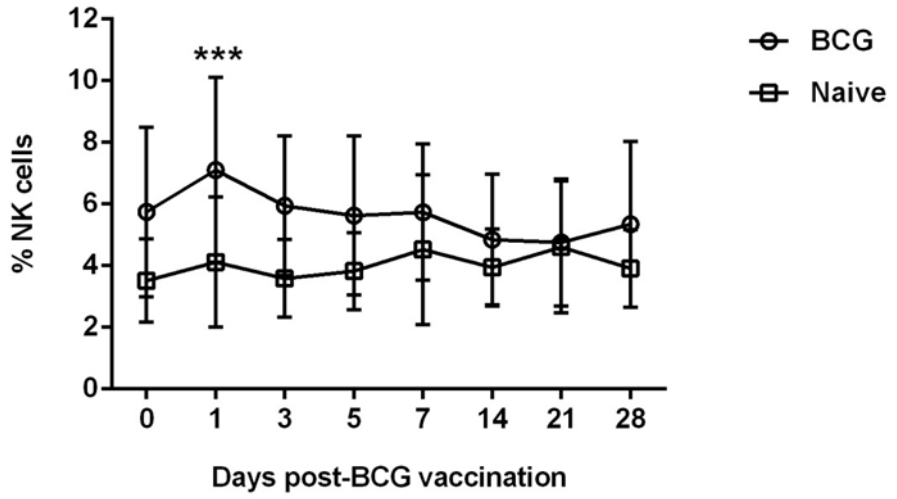


Figure 4.2 Percentage of NK cells and subsets present in the blood following BCG vaccination

Lymphocytes (fresh) were isolated from PB at Day 0, 1, 3, 5, 7, 14, 21 and 28 post-BCG vaccination or from age-matched, non-vaccinated calves and labelled with mAbs to NKp46 and CD2 and analysed by flow cytometry. NK cells and NK cell subsets were identified using the gating strategies shown in Figure 3.2.1. Pooled data from 12 BCG-vaccinated calves (circles) and 9 naïve calves (squares) represents the average percentage of NK cells ± SD present within the total lymphocyte population at various time points post-BCG vaccination. Data were non-normally distributed ($p<0.05$), therefore data was log transformed prior to assessment of significance using GLM and paired t-tests. $p<0.001$***.

4.2.3 Phenotype of NK cells following BCG vaccination

Similar to investigations detailed in Chapter 3, NK cell expression of CD25 was used as a measure of NK cell activation following BCG vaccination. Lymphocytes were labelled with mAbs to NKp46, CD2 and CD25 and analysed by flow cytometry. The percentage of CD25+ NK cells present in the blood increased on Days 1, 3, 5 and 7 post-BCG vaccination, however these changes were not significantly different to the percentage of NK cells present pre-vaccination (Day 0). No significant differences in the activation of NK cells from non-vaccinated calves were noted (Figure 4.3). CD25 was equally expressed by CD2+ and CD2- subsets of NK cells (as illustrated in Figure 3.3.4), however no significant effect of BCG on the proportions of CD2+ and CD2- subsets of NK cells expressing CD25 were evident throughout the vaccination experiment (data not shown).

4.2.4 NK cell production of IFN-γ following BCG vaccination

An important effector function of NK cells is the secretion of immunoregulatory cytokines, particularly IFN-γ. Thus, to assess the ability of NK cells to produce IFN-γ following BCG vaccination, intracellular cytokine staining (section 2.14) was employed. Lymphocytes were labelled with mAbs to NKp46 and IFN-γ and cells were analysed by flow cytometry. Following BCG vaccination, the percentage of IFN-γ+ NK cells increased at both early and later time points post-vaccination. However, non-specific production of IFN-γ by NK cells from naïve calves was also evident therefore making these results difficult to interpret (Figure 4.4).

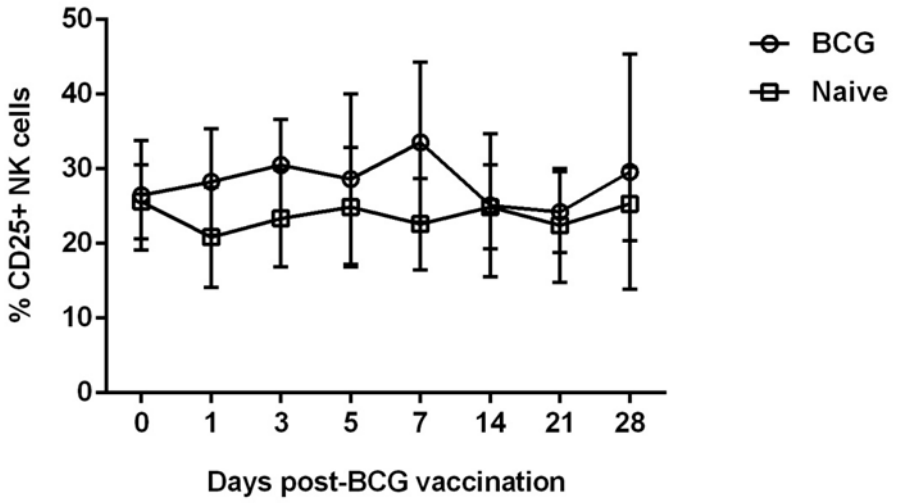


Figure 4.3 Phenotype of NK cells following BCG vaccination

Lymphocytes (fresh) were isolated from PB at Day 0, 1, 3, 5, 7, 14, 21 and 28 post-BCG vaccination or from age-matched, non-vaccinated calves and labelled with mAbs to NKp46, CD2 and CD25 and analysed by flow cytometry. CD25+ NK cells were identified using the gating strategy illustrated in Figure 3.3.1. Pooled data from 12 BCG-vaccinated calves (circles) and 9 non-vaccinated calves (squares) represents the average percentage of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population at various time points post BCG-vaccination. Data were normally distributed ($p>0.05$) and significance was assessed using GLM and paired t tests. No significant differences were noted.

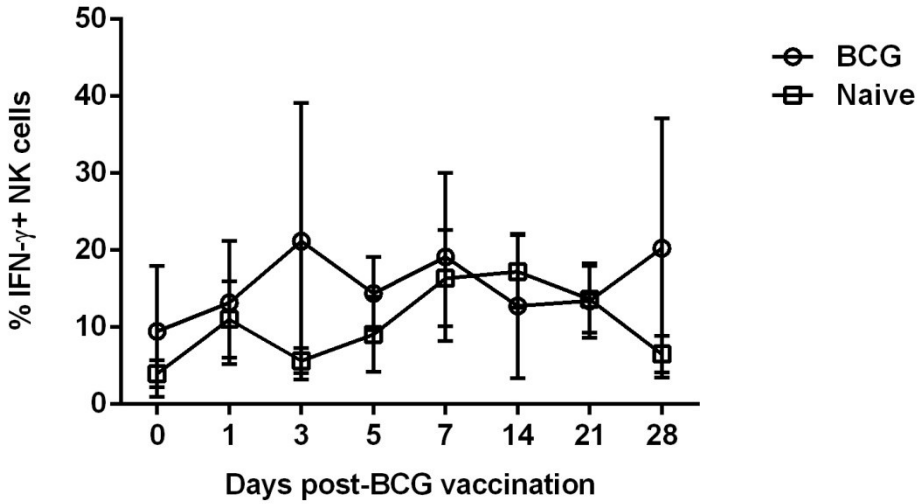


Figure 4.4 NK cell production of IFN-γ following BCG vaccination

Lymphocytes (fresh) were isolated from PB at Day 0, 1, 3, 5, 7, 14, 21 and 28 post-BCG vaccination or from age-matched, non-vaccinated calves and labelled with mAbs to NKp46 and IFN-γ and analysed by flow cytometry. Pooled data from 12 BCG-vaccinated calves (circles) and 9 non-vaccinated calves (squares) illustrates the average percentage of IFN-γ+ NK cells ± SD within the total gated NKp46+ NK cell population at various time points post-BCG vaccination.

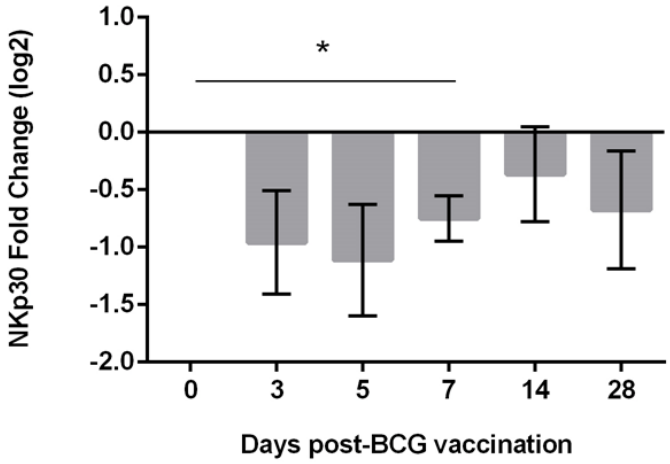
4.2.5 Expression of *NKp30* by NK cells following BCG vaccination

NK cells express a diverse array of activating and inhibitory receptors which together control their effector function and maintain self-tolerance. To evaluate the effect of BCG vaccination on the receptor repertoire of NK cells, three six-week old calves were vaccinated subcutaneously with 0.5ml BCG Danish SSI and NK cells were isolated at days 0, 3, 5, 7, 14 and 28 post-BCG vaccination. In parallel, NK cells were also isolated from three age-matched, non-vaccinated control calves. RNA was isolated, reverse transcribed and NK cell expression of the activating receptor, NKp30 was assessed at various time points post-vaccination by qPCR. Expression of *NKp30* by NK cells was significantly down-regulated at day 7 post-BCG vaccination ($p=0.037$), compared with Day 0, most likely due to the tight errors bars at day 7 post-BCG (Figure 4.5.1). No significant changes were noted in the expression of *NKp30* by NK cells derived from non-vaccinated animals (Figure 4.5.2).

4.2.6 NK cell expression of *KLRC1.2*, *KLRC2.1* and *KLRC2.2* following BCG vaccination

To further characterise the receptor repertoire of NK cells following BCG vaccination, preliminary experiments sought to compare the expression of three members of the KLRC (NKG2) family: KLRC1.2 (NKG2A: inhibitory receptor), KLRC2.1 (NKG2C: activating receptor) and KLRC2.2 (NKG2C: activating receptor) between naïve and BCG-vaccinated calves. Whilst it was difficult to quantify the exact amount of each gene expressed at each day post-BCG vaccination using PCR, there appeared to be an overall increased expression of *KLRC1.2*, *KLRC2.1* and *KLRC2.2* in BCG-vaccinated calves compared with naïve calves, denoted by the stronger bands in the vaccinated calves (202878, 302879 and 402880) (Figure 4.6).

4.5.1



4.5.2

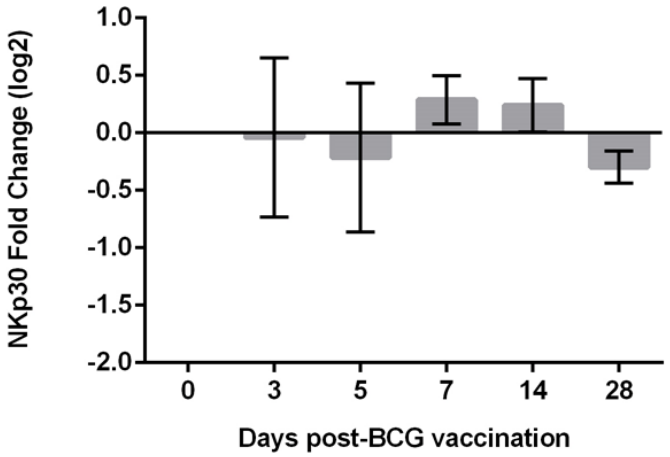


Figure 4.5 Expression of *NKp30* by NK cells following BCG vaccination

NK cells were isolated from the PB of three BCG-vaccinated and three age-matched, non-vaccinated calves on day 0, 3, 5, 7, 14 and 28 post-vaccination. RNA was extracted, reverse transcribed and subject to quantitative PCR analysis for the expression of *NKp30*. Each sample was tested in duplicate and normalised to two reference genes, *ATP5B* and *EIF2B2* and analysed using the comparative CT method. Data represents the fold change (Log2) of the CT values of *NKp30* expression ± SD at day 3, 5, 7, 14 and 28 post-vaccination, compared with day 0 by NK cells from BCG-vaccinated (Figure 4.5.1) and non-vaccinated (Figure 4.5.2) calves. Data were normally distributed ($p>0.05$) and significance was assessed using paired t-tests. $p<0.05$*.

Figure 4.6

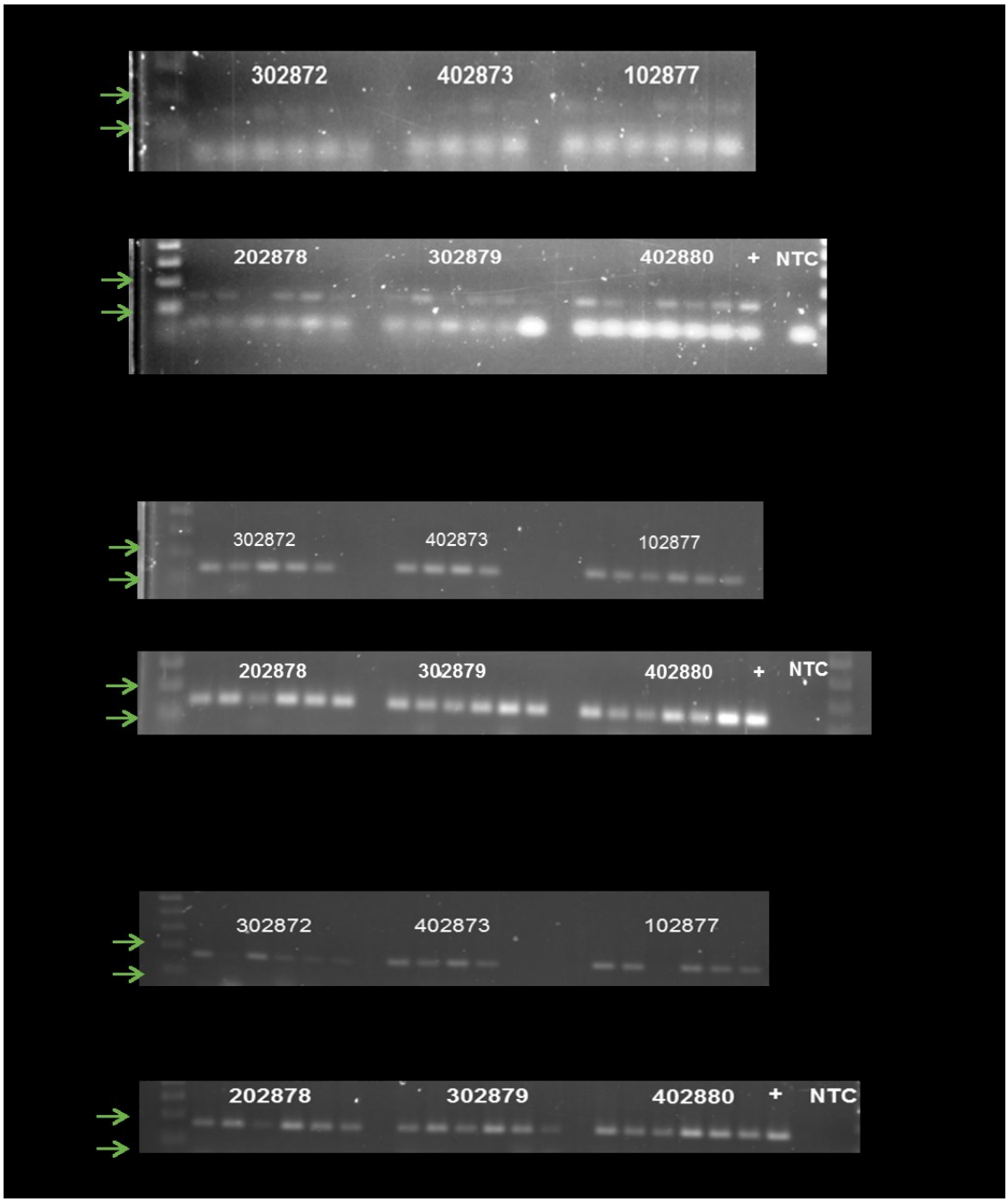

302872
402873
102877
202878
302879
402880
+
NTC
302872
402873
102877
202878
302879
402880
+
NTC
302872
402873
102877
202878
302879
402880
+
NTC

Figure 4.6 NK cell expression of *KLRC1.2*, *KLRC2.1* and *KLRC2.2* following BCG vaccination

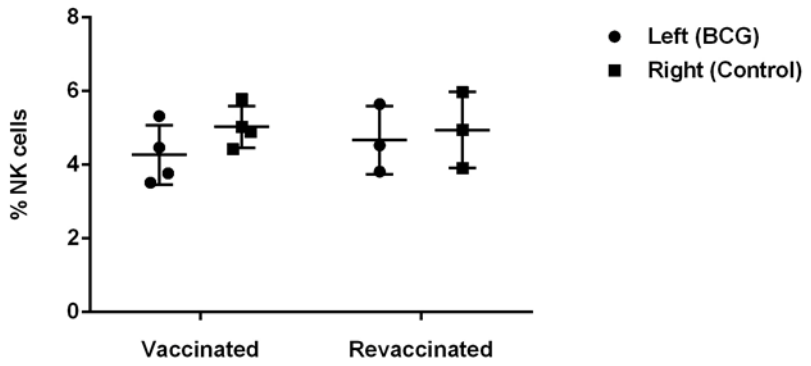
NK cells were isolated from the PB of three BCG-vaccinated and three age-matched, non-vaccinated calves on day 0, 3, 5, 7, 14 and 28 post-vaccination. RNA was extracted, reverse transcribed and subject to PCR analysis for the expression of *KLRC1.2*, *KLRC2.1* and *KLRC2.2*. cDNA positive for the gene of interest was used as the positive control (+) and water as the negative control (NTC). PCR products were loaded on a 2% agarose gel, alongside a 1Kb PLUS DNA ladder. Expected band sizes for each gene were as follows: *KLRC1.2* (140bp), *KLRC2.1* (140bp) and *KLRC2.2* (180bp). It should be noted that day 3 and 28 samples are missing from naïve animal, 402873.

4.2.7 Effect of BCG on LN derived NK cells 24 hours post-vaccination

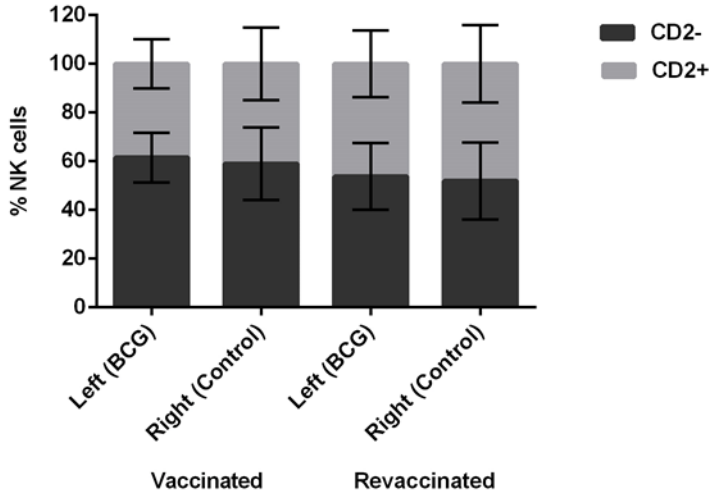
Following characterisation of the frequency, phenotype and function of PB derived NK cells after BCG vaccination of neonatal calves, the frequency and phenotype of NK cells within PSLNs draining the site of BCG vaccination were defined. To assess recruitment of NK cells to LNs *in vivo*, 0.5ml BCG Danish SSI was administered subcutaneously to the left shoulder of 6 month old, non-vaccinated calves (referred to as 'vaccinated' herein) or to age-matched calves which had been previously BCG-vaccinated at 6 weeks of age (referred to as 'revaccinated' herein) and draining PSLNs were then excised 24 hours post-vaccination. PSLNs from the right shoulder (non-vaccinated) of each calf served as an internal control.

To assess if the percentage of NK cells, or the percentage of NK cell subsets, within the LNs was altered following BCG vaccination, lymphocytes were labelled with mAbs to NKp46, CD3 and CD2 analysed by flow cytometry. No significant differences were noted in the percentage of NK cells present (Figure 4.7.1) or the distribution of the CD2+ and CD2- subsets (Figure 4.7.2) between the left and right LNs from the vaccinated or revaccinated calves at 24 hours post-BCG vaccination. To assess if BCG vaccination altered the activation status of NK cells within the LNs draining the site of vaccination, the percentage of CD25+ NK cells present in the left and right PSLNs of vaccinated or revaccinated calves at 24 hours post-vaccination was defined by labelling lymphocytes with mAbs to NKp46, CD2 and CD25 and analysing by flow cytometry. Despite a high percentage of LN derived NK cells expressing CD25 (as illustrated in Figure 3.3), there were no significant differences evident at 24 hours post-BCG in the left or right LNs in either vaccinated or revaccinated calves (Figure 4.7.3). No differences were noted in the expression of CD25, represented by the MFI (data not shown).

4.7.1


% NK cells
8
6
4
2
0
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

4.7.2


% NK cells
120
100
80
60
40
20
0
Left (BCG)
Right (Control)
Left (BCG)
Right (Control)
Vaccinated
Revaccinated
CD2-
CD2+

4.7.3

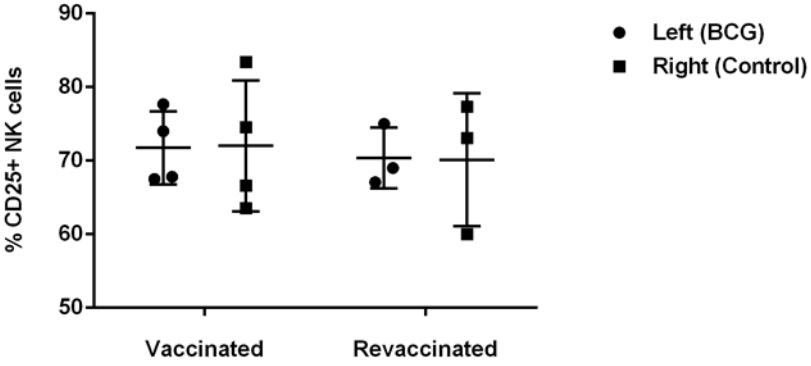

% CD25+ NK cells
90
80
70
60
50
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

Figure 4.7 Effect of BCG on LN derived NK cells 24 hours post-vaccination

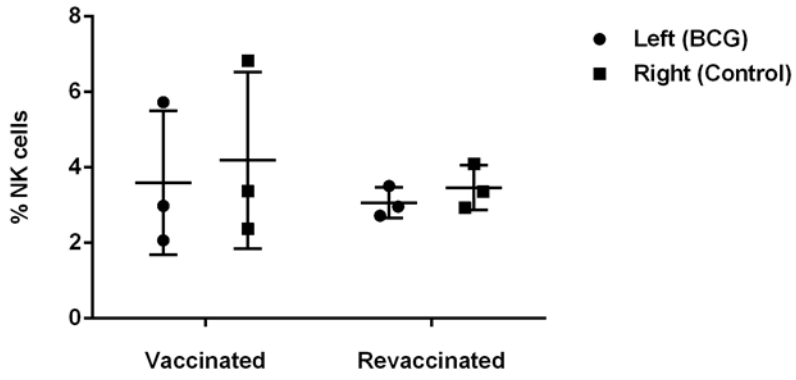
Lymphocytes (fresh) derived from the left (BCG) and right (control) LNs at 24 hours post-BCG vaccination were labelled with mAbs to NKp46, CD3 and CD2 and analysed by flow cytometry. Pooled data from the left (circles) and right (squares) LNs of four vaccinated and three revaccinated calves represents the average percentage of NKp46+ CD3- NK cells ± SD (Figure 4.7.1). Pooled data from the left and right LNs of four vaccinated and three revaccinated calves illustrates the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.7.2). Pooled data from the left (circles) and right (squares) LNs of four vaccinated and three revaccinated calves illustrates the average percentage of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.7.3). Data were normally distributed ($p>0.05$) and significance was assessed using two sample t-tests. No significant differences were noted.

4.2.8 Effect of BCG on LN derived NK cells 48 hours post-vaccination

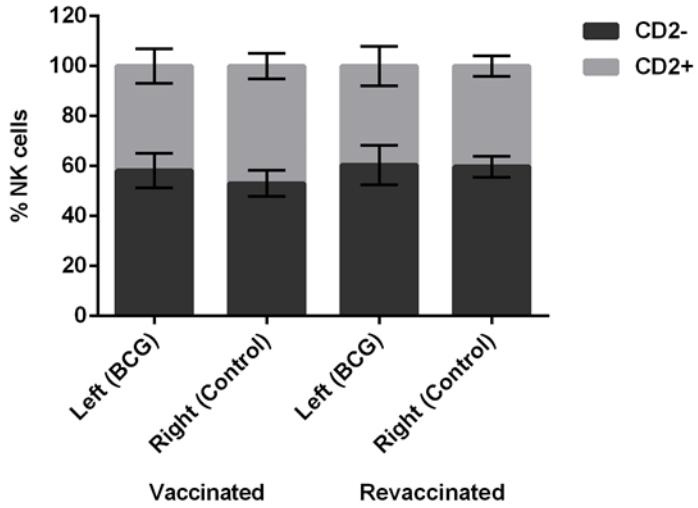
Vaccination of calves with BCG had no significant effect on the percentage, subset distribution or phenotype of LN derived NK cells at 24 hours post-vaccination, therefore LNs were excised 48 hours following vaccination to decipher if BCG altered the properties of NK cells within the draining LNs at this later time point. As described in section 4.2.7, 0.5ml BCG Danish SSI was administered subcutaneously to the left shoulder of three six month old, non-vaccinated calves ('vaccinated') or age-matched calves which had been previously BCG-vaccinated at six weeks of age ('revaccinated') and draining LNs were then excised 48 hours after vaccination. LNs from the right shoulder (non-vaccinated) of each calf served as an internal control.

To assess the percentage, subset distribution and activation status of NK cells in the draining LNs at 48 hours post-BCG, lymphocytes were labelled with mAbs to NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. Similar to findings at 24 hours post-BCG (Figure 4.7), no significant differences were evident in the percentage of NK cells (Figure 4.8.1), distribution of CD2+ and CD2- subsets (Figure 4.8.2) and percentage of CD25+ NK cells (Figure 4.8.3) present in the left and right LNs in vaccinated and revaccinated calves 48 hours after vaccination. Furthermore, no differences were noted in the expression of CD25, represented by the MFI (data not shown). Similar to findings in Figure 3.3.4, CD2- NK cells were the predominant subset of NK cells expressing CD25, however no differences were noted in the percentage of CD2- and CD2+ NK cells expressing CD25 between the left and right LNs in vaccinated or revaccinated calves at 48 hours post-vaccination (data not shown).

4.8.1


% NK cells
8
6
4
2
0
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

4.8.2


% NK cells
120
100
80
60
40
20
0
Left (BCG)
Right (Control)
Left (BCG)
Right (Control)
Vaccinated
Revaccinated
CD2-
CD2+

4.8.3

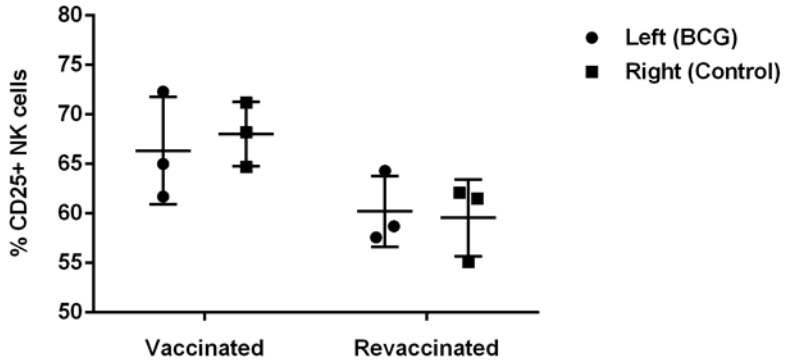

% CD25+ NK cells
80
75
70
65
60
55
50
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

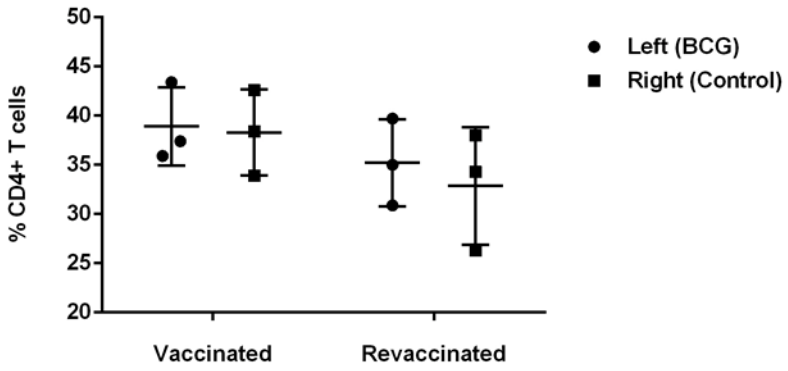
Figure 4.8 Effect of BCG on LN derived NK cells 48 hours post-vaccination

Lymphocytes (fresh) derived from the left (BCG) and right (control) LNs were labelled with mAbs to NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. Pooled data from the left (circles) and right (squares) LNs of three vaccinated and three revaccinated calves indicate the average percentage of NKp46+ CD3- NK cells ± SD (Figure 4.8.1). Pooled data from the left and right LNs of three vaccinated and three revaccinated calves represents the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.8.2). Pooled data from the left (circles) and right (squares) LNs of three vaccinated and three revaccinated calves illustrate the average percentage of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.8.3). Data were normally distributed ($p>0.05$) and significance was assessed using two sample t-tests. No significant differences were noted.

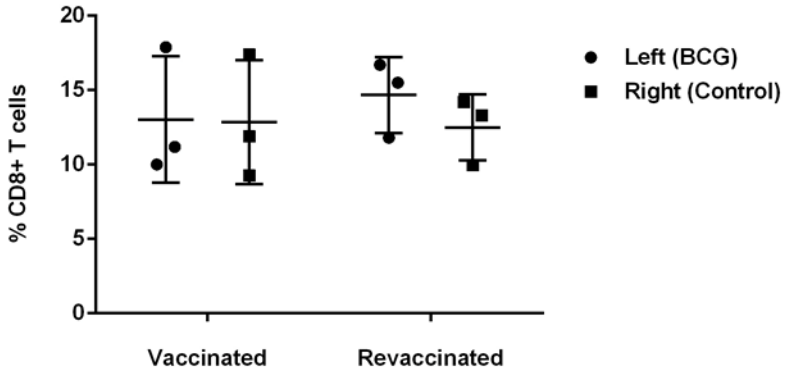
4.2.9 Effect of BCG on LN derived T cells 48 hours post-BCG vaccination

Data presented thus far have illustrated no significant effect of BCG on NK cell frequency or phenotype within the LNs draining the site of vaccination. Therefore, the effect of BCG vaccination on the percentage of CD4+ T cells, CD8+ T cells and WC1+ γδ T cells was assessed to determine if BCG affected the frequency of other lymphocyte populations within the LNs. Lymphocytes were labelled with CD3/CD4, CD3/CD8 and CD3/WC1 to identify CD4+ T cells, CD8+ T cells and WC1+ γδ T cells respectively. Cells were then analysed by flow cytometry. In parallel with the absence of effect of BCG on NK cell frequency and phenotype, the percentage of CD4+ T cells (Figure 4.9.1), CD8+ T cells (Figure 4.9.2) and WC1+ γδ T cells (Figure 4.9.3) were unchanged in the right control LNs, compared with the left LNs draining the site of vaccination, from both vaccinated and revaccinated calves.

4.9.1


% CD4+ T cells
50
45
40
35
30
25
20
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

4.9.2


% CD8+ T cells
20
15
10
5
0
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

4.9.3

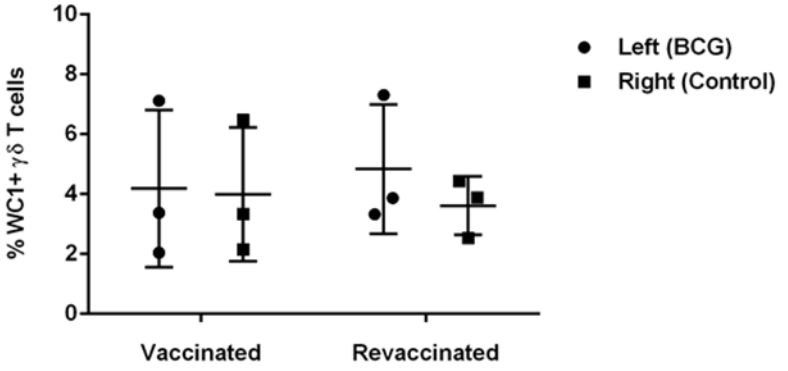

% WC1+ γδ T cells
10
8
6
4
2
0
Vaccinated
Revaccinated
Left (BCG)
Right (Control)

Figure 4.9 Effect of BCG on LN derived T cells at 48 hours post-BCG vaccination

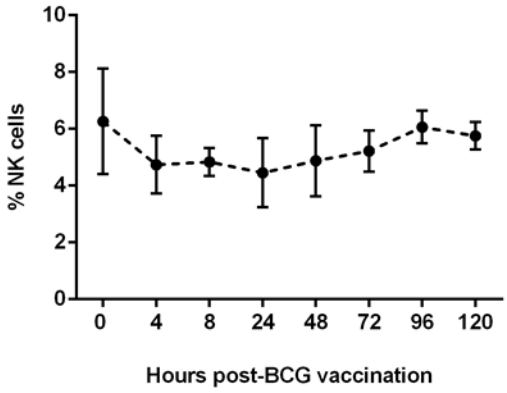
Lymphocytes (fresh) derived from the left (BCG) and right (control) LNs were labelled with mAbs to CD3, CD4, CD8 and WC1 and analysed by flow cytometry. Pooled data from the left (circles) and right (squares) LNs of three vaccinated and three revaccinated calves indicate the average percentage of CD3+CD4+ T cells ± SD (Figure 4.9.1), CD3+CD8+ T cells ± SD (Figure 4.9.2) and CD3+WC1+ γδ T cells ± SD (Figure 4.9.3) present at 48 hours post-BCG vaccination. Data were normally distributed ($p>0.05$) and significance was assessed using two sample t-tests. No significant differences were noted.

4.2.10 Percentage and phenotype of AL derived NK cells following BCG vaccination

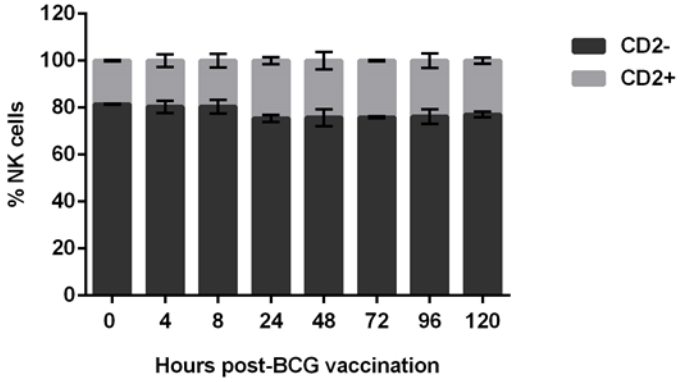
BCG vaccination had no effect on the percentage, subset distribution or phenotype of NK cells within draining PSLNs either at 24 or 48 hours post-vaccination (Figures 4.7 and 4.8), therefore the pseudo-afferent lymphatic cannulation model was utilised to study the effect of BCG vaccination on NK cells within draining AL in preliminary experiments using two pseudo-afferent lymphatic cannulated calves aged 6 months. 0.5ml of BCG Danish SSI was inoculated subcutaneously above the cannulation site (section 2.2.1) and afferent lymph was collected at 0, 4, 8, 24, 48, 72, 96 and 120 hours post-vaccination to establish firstly, at what time point post-vaccination NK cells may be arriving in the LNs via afferent lymphatic vessels and secondly, whether BCG alters the phenotype of AL derived NK cells.

To define the percentage, subset distribution and phenotype of AL derived NK cells after BCG vaccination, lymphocytes were labelled with mAbs to NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. The percentage of NK cells decreased within the first 4 hours post-BCG and reached the lowest percentage at 24 hours post-BCG. NK cell frequency then increased from 24 hours post-vaccination and returned to baseline levels at 96 hours post-BCG (Figure 4.10.1). CD2- NK cells decreased within AL over the course of the experiment (Figure 4.10.2). The percentage of CD25+ NK cells increased within AL within the first 4 hours post-BCG and remained steady throughout the remainder of the experiment (Figure 4.10.3) with the percentage of CD2- CD25+ NK cells increasing over time (Figure 4.10.4). A similar pattern was seen in the percentage of CD25+ NK cells over time, as represented by the MFI (data not shown).

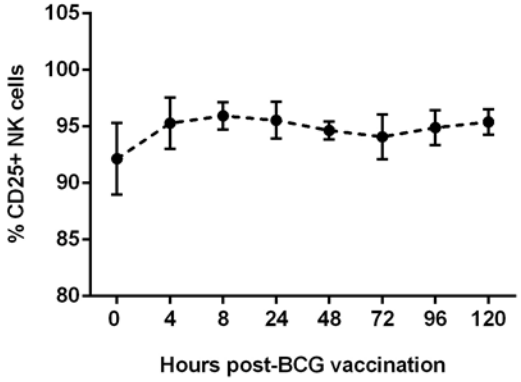
4.10.1



4.10.2



4.10.3



4.10.4

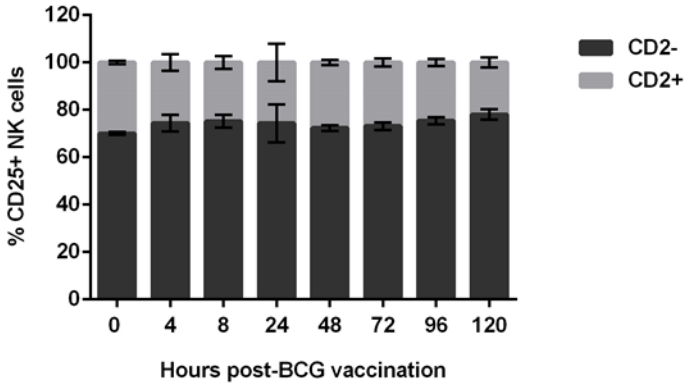


Figure 4.10 Percentage and phenotype of AL derived NK cells following BCG vaccination

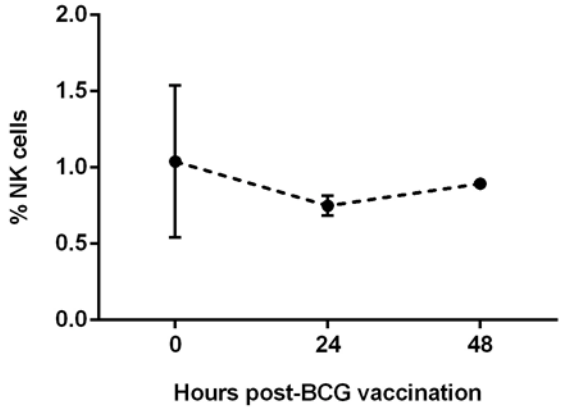
Lymphocytes derived from AL (fresh) of two BCG-vaccinated pseudo-afferent lymphatic cannulated calves were labelled with mAbs for NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. Pooled data from two calves show: the average percentage of NKp46+ CD3- NK cells ± SD (Figure 4.10.1); the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.10.2); the average percentage of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.10.3); the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ CD25+ population present within AL at hours 0, 4, 8, 24, 48, 72, 96 and 120 hours post-BCG vaccination. Statistical analysis was not performed due to the preliminary nature of the experiment (n=2).

4.2.11 Percentage and phenotype of EL derived NK cells following BCG vaccination

Data presented previously in this thesis (Figure 3.8) demonstrated that bovine NK cells were present in the efferent lymphatic vessel draining the skin in steady-state conditions, therefore the effect of BCG vaccination on NK cells with EL was addressed. To assess egress of NK cells from the LNs draining the site of BCG vaccination, three 6 month old efferent lymphatic cannulated calves were vaccinated subcutaneously with BCG Danish SSI at APHA and efferent lymph was collected at 0, 24 and 48 hours post-BCG vaccination (section 2.2.3).

To define the percentage, subset distribution and phenotype of EL derived NK cells after vaccination, lymphocytes were labelled with mAbs to NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. No significant differences were observed in the percentage of NK cells (Figure 4.11.1) or the percentage of NK cell subsets (Figure 4.11.2) present within EL at 24 or 48 hours post-BCG compared with pre-vaccination. Despite a decrease in the percentage (Figure 4.11.3) and MFI (Figure 4.11.4) of CD25+ NK cells over the course of the experiment, this was not significant.

4.11.1



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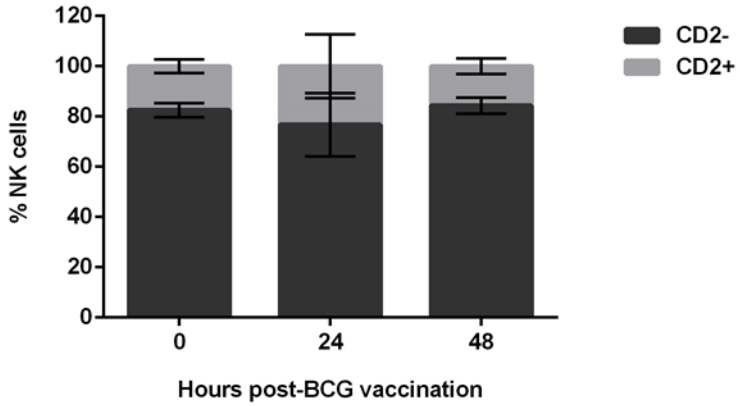

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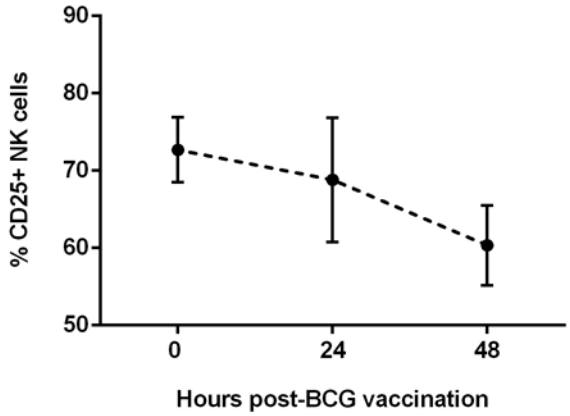

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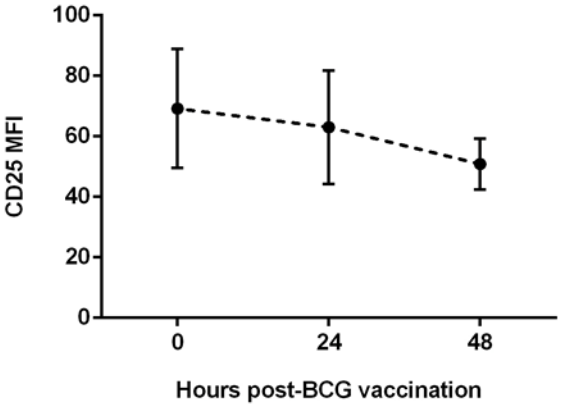


Figure 4.11 Percentage and phenotype of EL derived NK cells following BCG vaccination

Lymphocytes derived from EL (frozen) of three BCG-vaccinated efferent lymphatic cannulated calves were labelled with mAbs for NKp46, CD3, CD2 and CD25 and analysed by flow cytometry. Pooled data from three calves show: the average percentage of NKp46+ CD3- NK cells ± SD (Figure 4.11.1); the average percentage of CD2+ (lighter bars) and CD2- (darker bars) NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.11.2); the average percentage of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.11.3); the average MFI of CD25+ NK cells ± SD within the total gated NKp46+ NK cell population (Figure 4.11.4) present within EL at hours 0, 24 and 48 post-BCG vaccination. Data were normally distributed ($p>0.05$) and significance was assessed using two sample t-tests. No significant differences were noted.

4.3 Discussion

The immune response following BCG vaccination of neonatal calves that results in protection against bTB has not yet been fully defined. The frequency of NK cells is augmented in neonatal calves (Graham et al., 2009, Kulberg et al., 2004) and since NK cells contribute to anti-mycobacterial immunity in humans and mice (Esin and Batoni, 2015), NK cell responses post-BCG vaccination of neonatal calves were the primary focus of this study. Six week old neonatal calves responded to BCG vaccination by producing antigen-specific IFN-γ, with peak responses around four weeks post-vaccination, confirming successful delivery of BCG (Figure 4.1). Following BCG vaccination of neonatal calves, there was a significant increase in the percentage of NK cells present in the circulation during the first 24 hours post-BCG (Figure 4.2). Whether this early expansion of NK cells within the blood post-vaccination is due to generation of NK cells from the bone marrow, or from proliferation of NK cells within the circulation remains to be defined. Furthermore, this increase in the percentage of NK cells may be due to a decrease in another cell population and to determine if this was the case, changes in absolute numbers would have to be defined. This early increase in NK cell frequency post-BCG was followed by a non-significant decrease in the percentage of NK cells present in the blood from day 7 post-vaccination, which may reflect movement of NK cells out of the circulation and to the site of vaccination. No differences were noted in the proportions of CD2+ and CD2- NK cells in both BCG-vaccinated and non-vaccinated calves (data not shown), therefore the observed increased in NK cells present at day 1 post-BCG was not attributed to a particular subset of NK cells. Zufferey *et al* assessed the contribution of innate effector cells such as NK cells and γδ T cells during the immune response elicited by BCG immunisation of infants (Zufferey et al., 2013). They demonstrated that NK cells were a significant source of BCG-specific IFN-γ following vaccination, with NK cells contributing to more than half of the IFN-γ-expressing cells in infants vaccinated at birth and at 2 months of age. Following BCG vaccination of adults, the proportion of NK cell subsets, expression of cell surface molecules or production of IFN-γ remained unaltered at 2 weeks and 3 months post-BCG. However, production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) was significantly increased after *ex vivo* stimulation with *M.tb, Candida albicans* and *Staphylococcus aureus*. Therefore

in this particular study, BCG induced non-specific priming of NK cells (Kleinnijenhuis et al., 2014). WC1+ γδ T cells are also present in increased levels in young calves, and following BCG vaccination of calves, the percentage of WC1+ γδ T cells increased in the PB post-BCG alongside increased production of IFN-γ (Buza et al., 2009). Therefore calves may respond to BCG vaccination in a similar manner to humans.

The activation status of NK cells derived from the blood was generally low, compared with those NK cells found in AL, LNs and EL (as described in Figure 3.3) and BCG had no significant effect on the expression of CD25 by NK cells post-vaccination (Figure 4.3). Similarly, the function of NK cells post-BCG vaccination was difficult to interpret due to non-specific production of IFN-γ by NK cells from naïve calves (Figure 4.4). NK cells from young animals are a source of non-specific IFN-γ and for this reason the IFN-γ diagnostic test cannot be used in animals less than 6 months old (Olsen et al., 2005).

Preliminary experiments suggested that BCG vaccination of neonatal calves altered NK cell receptor usage (Figure 4.5 and Figure 4.6). NK cell gene expression of *NKp30* was significantly down-regulated following BCG vaccination (Figure 4.5). Similar to the present study, mRNA expression of *NKp30* by NK cells was significantly down-regulated following challenge with Bovine Respiratory Syncytial Virus (BRSV) (Alasdair Allan, PhD thesis, Royal Veterinary College, 2015). Taken together, this may reflect stimulation of bovine NK cells by pathogens such as BCG and BRSV, resulting in down-regulation of *NKp30* mRNA *in vivo*. NK cell function and maintenance of self-tolerance is determined by a complex interplay between activating and inhibitory receptors, therefore it would be expected that down-regulation of NKp30 would be coupled with up-regulation of other NK cell receptors. This maintains a delicate balance between activation and inhibition and also regulates the activity of NK cells to prevent damage from NK cell cytotoxicity or overexpression of cytokines. The *KIR* and *CD94/NKG2* (*KLRD/KLRC*) receptor gene families are significantly expanded in cattle (Figure 1.3) with the *CD94/NKG2* (*KLRD/KLRC*) family containing 2 *CD94* genes, 7 *NKG2A* genes and 2 *NKG2C* genes. Thus, the expression of *KLRC1.2* (*NKG2A* - inhibitory receptor), *KLRC2.1* (*NKG2C* - activating receptor) and *KLRC2.2* (*NKG2C* - activating receptor) by BCG-vaccinated and non-

vaccinated calves was assessed by PCR. Preliminary results showed an overall increased expression of *KLRC1.2* (*NKG2A*), *KLRC2.1* (*NKG2C*) and *KLRC2.2* (*NKG2C*) in BCG-vaccinated calves, compared with non-vaccinated calves (Figure 4.6). Again this may reflect the complex interplay of NK cell receptors expressed at any one time with up regulation of both inhibitory and activating genes following vaccination. Nevertheless, to gain a wider understanding of the NK cell receptor repertoire following BCG vaccination of neonatal calves and to confirm the PCR results described in Figure 4.6, qPCR assays would have to be designed.

To assess the capacity of NK cells as correlates of protection during BCG-induced protective immunity to *M. bovis* infection in cattle, NK cell responses would have to be analysed both during BCG vaccination and then following challenge with *M. bovis*. Challenge studies in large animals are very expensive, however it was proposed recently that a BCG challenge model could be utilised to negate the requirement for costly containment level three challenge experiments (Villarreal-Ramos et al., 2014). The authors hypothesised that a TB vaccine capable of conferring protection against *M. bovis* should also be able to induce immunity against the live attenuated strain of *M. bovis*, BCG. Calves vaccinated subcutaneously with BCG Danish SSI were protected from challenge with a different strain of BCG, BCG Tokyo which was delivered intranodally. This BCG challenge model has also been proposed to test TB vaccine candidates in humans (Minassian et al., 2012, Harris et al., 2014). To definitively demonstrate a functional role of NK cells during BCG-induced protection, NK cells would have to be deleted *in vivo* by administration of mAb specific for NKp46 prior to BCG vaccination and challenge with *M. bovis*. Studies have depleted γδ T cells (Kennedy et al., 2002) or CD8+ T cells (Villarreal-Ramos et al., 2003) from the circulation of calves either prior to *M. bovis* infection or after *M. bovis* infection and then assessed the effect of depletion on infection. Both of these experimental approaches would serve as excellent systems to address the role of NK cells in BCG-induced protection but, to date, neither of these approaches have been assessed in any published studies.

Within the BCG vaccination experiments described thus far, BCG was delivered subcutaneously therefore analysis of NK cell responses occurring in the afferent

lymphatic vessels, PSLNs and efferent lymphatic vessel which specifically drain the site of vaccination may improve our knowledge of the immune response induced by BCG. It was demonstrated in Chapter 3 that highly activated, CD2- NK cells could migrate into skin draining LNs via afferent lymphatic vessels in steady-state conditions. Afferent lymphatic cannulation is an excellent model to study innate immune responses during vaccination or infection therefore this model was utilised to assess the recruitment of NK cells to the LNs via the afferent lymphatic vessel, in the context of BCG vaccination. This preliminary experiment, with two BCG-vaccinated pseudo-afferent lymphatic cannulated calves, showed a transient decrease in the percentage of NK cells present within AL at 4 hours post-BCG vaccination with the lowest levels of NK cells present at 24 hours post-vaccination. This decrease may be due to an increase in another cell population and therefore the absolute number of NK cells within AL following BCG vaccination would have to be defined. The percentage of NK cells within AL returned to pre-vaccination levels by 96 hours following vaccination (Figure 4.10.1). A decrease in the percentage of CD2- NK cells in AL was evident over time and may reflect selective movement of CD2- NK cells through AL and into LNs post-BCG vaccination (Figure 4.10.2). The decrease in the percentage of NK cells present within 4 hours post-BCG was coupled with an increase in the activation of AL derived NK cells within this period (Figure 4.10.3), which was attributed to the CD2- subset of NK cells (Figure 4.10.4). Following subcutaneous administration of GFP-labelled recombinant human replication-defective human adenovirus 5 (rhuAdV5) and water-in-oil adjuvant, the frequency of migratory DCs in AL increased within 4 hours and peaked at 15 hours post-inoculation, indicating that alterations to populations of innate immune cells occurs early post-vaccination (Cubillos-Zapata et al., 2011). Overall, data described in Figure 4.2.10 provide novel, preliminary evidence that NK cell frequency and phenotype within afferent lymph is altered following BCG vaccination.

Left (BCG) and right (control) PSLNs draining the site of vaccination from vaccinated or revaccinated calves were removed either 24 hours or 48 hours post-BCG vaccination and NK cells were analysed for differences in frequency or phenotype. No differences in the percentage of NK cells present in the LNs draining the site of BCG vaccination were evident at either 24 hours or 48 hours post-vaccination in both

vaccinated and revaccinated calves (Figure 4.7 and Figure 4.8). Furthermore, LNs were also weighed and no differences were noted between the left, vaccinated LNs and the right, control LNs (data not shown). There may be a few possible explanations to describe the lack of alterations to NK cell frequency or phenotype within the nodes following BCG vaccination. Firstly, it may be that NK cell frequency and phenotype is altered at a time-point earlier or later than 24 or 48 hours post-BCG vaccination. For example, experiments presented in Figure 4.10.1 showed changes in AL derived NK cells as early as 4 hours post-BCG vaccination. During studies in mice, NK cells entered LNs 2 days after injection with LPS-matured DCs or adjuvants such as R848 and Ribi. In this study, it was shown that NK cells were trafficking from the blood into the LNs (following intravenous injection of LPS-DCs, R848 and Ribi), however NK cells were not recovered from the LNs when LPS-DCs, R848 and Ribi were administered subcutaneously (Martin-Fontecha et al., 2004). Secondly, NK cells are rapidly recruited to murine LNs following vaccination or infection (Martin-Fontecha et al., 2004, Lucas et al., 2007) however, unlike bovine and human LNs, mouse LNs contain low frequencies of NK cells in steady-state conditions. Therefore, an influx of NK cells during inflammation may be more pronounced or easy to detect in mice, compared with cattle or humans. Alternatively, NK cells may be redistributed within LNs to areas where accessory cells such DCs are located or vaccine-induced NK cells may displace resident NK cells from the LNs. Both of these hypotheses would result in the percentage of NK cells within BCG draining LNs remaining relatively constant. Finally, it may be that BCG affects both the left PSLN draining the site of vaccination and the right PSLN and therefore may be the reason why there were no differences between NK cells in the left and right nodes.

Evidence presented in Chapter 3 illustrated that NK cells are present within bovine efferent lymph and therefore indicated that NK cells may egress from the LNs to return to circulation in steady-state conditions. Following BCG vaccination, there were no significant differences in the percentage of NK cells present in EL at 24 or 48 hours post-BCG, compared with pre-vaccination (0 hours). The percentage of NK cells present at time 0 in the three calves tested was very variable as denoted by the large error bars (Figure 4.11.1). Nevertheless, the presence of NK cells within EL at 24 and 48 hours post-BCG vaccination implies that BCG does not prevent egress of NK cells

from the LNs. It is thought that in response to antigen or infection that there is a shutdown in lymphocyte output that lasts up to 24 hours (as reviewed by (Haig et al., 1999)), however NK cells were present in EL at both 24 and 48 hours post-vaccination. CD2- NK cells remained the predominant subset of NK cells present in EL throughout the course of the experiment (Figure 4.11.2). A non-significant decrease in the activation of NK cells was noted, as illustrated by both percentage of CD25+ NK cells and MFI (Figure 4.11.3). This experiment would need to be repeated in more calves to define the effect of BCG on NK cells egressing from the LNs by the efferent lymphatic vessel.

Injecting fluorescently labelled NK cells would be an optimal method to track the movement of NK cells from the site of BCG vaccination, through skin draining afferent lymphatic vessels, LNs and their subsequent egress from the efferent lymphatic vessel. This would also allow confirmation that it is the CD2- subset of NK cells which are selectively moving in and out of LNs via the lymphatics.

To conclude, BCG vaccination of neonatal calves resulted in an increased percentage of NK cells within the circulation, and alterations to the receptor repertoire of bovine NK cells. BCG had no effect on NK cells within LNs draining the site of vaccination at either 24 or 48 hours post-vaccination. Preliminary experiments showed alterations to the frequency and phenotype of NK cells within afferent lymphatic vessels following vaccination. Overall, data presented in this Chapter suggests a potential role for NK cells during bovine anti-mycobacterial responses, thus supporting the hypothesis.

Abstract

NK cells play a role in shaping adaptive immune responses through interactions with DCs therefore interactions between NK cells and DCs in the context of BCG were investigated. DCs infected with BCG expressed significant levels of MHC class II and the co-stimulatory molecules CD40 and CD80, alongside augmented production of the Th1 polarising cytokine IL-12 when compared with uninfected DCs. As a result of *in vitro* co-culture with BCG-infected DCs, NK cells increased their expression of CD25 and the CD2- subset of NK cells was preferentially activated. NK cell effector function, as measured by production of IFN-γ, was also significantly enhanced following co-culture with BCG-infected DCs. This chapter provides evidence to demonstrate that NK cells phenotypically and functionally mature after interactions with DCs in the context of BCG. Furthermore, through the production of IFN-γ and IL-12 by NK cells and DCs respectively, this interaction may drive Th1 immune responses, which are important components of the immune response to mycobacteria.

5.1 Introduction

NK cells are traditionally regarded as cells of the innate immune system however as influential drivers of the adaptive immune response, NK cells can be viewed as an interface between innate and adaptive immunity. Early interactions between populations of innate immune cells, particularly NK cells and DCs, are important elements for determining the nature of the adaptive immune response. NK cells can be directly activated by BCG. For example, BCG can bind to human NK cells via NK cell expression of NKp44 to induce proliferation, cytotoxicity and production of IFN-γ (Esin et al., 2004, Esin et al., 2008). Similarly, expression of TLR2 by human NK cells permits direct recognition of BCG (Marcenaro et al., 2008). However, for the most part, NK cells require indirect activation through a complex interplay with populations of accessory cells, particularly DCs to become functionally mature (Lucas et al., 2007). The frequency, phenotype, function and receptor repertoire of NK cells following BCG vaccination of neonatal calves was described in Chapter 4. However, to fully understand NK cell responses during vaccination or infection, the role of accessory cells such as DCs and their relationship with NK cells must also be characterised.

Interactions between NK cells and DCs were first described *in vivo* in 1999 when it was demonstrated that DCs could modulate NK cell-mediated anti-tumour responses in mice (Fernandez et al., 1999). The bidirectional interplay between NK cells and DCs has been described in human and mouse models (Moretta, 2002, Walzer et al., 2005, Gerosa et al., 2005). This interplay has been the target for new therapies for cancer (Morandi et al., 2012, Shimizu and Fujii, 2009) and HIV (Altfeld et al., 2011). NK cells encounter DCs at sites of infection or inflammation and also within secondary lymphoid organs such as LNs (Cooper et al., 2004). For example, NK cells and DCs have been found in close contact in the lesions of patients with atopic eczema/dermatitis and were co-localised in the paracortex of the LNs (Ferlazzo et al., 2004). Co-localisation of NK cells, DC and T cells has also been demonstrated in LNs

(Walzer et al., 2007a, Lucas et al., 2007, Bajenoff et al., 2006), indicating a role for innate immune cell interactions in shaping T cell responses *in vivo*.

Similar to the interactions between APCs and T cells, interactions between NK cells and DCs are mediated by both soluble and contact-dependent signals. A central role for IL-12 in the cross-talk between NK cells and DCs is evident from a number of studies. IL-12 (IL-12p70) is a heterodimeric cytokine composed of a 40 kDa subunit (p40) and a 35 kDa subunit (p35) which drives Th1 polarisation of antigen-specific T cells, through the stimulation of IFN-γ (Macatonia et al., 1993). The importance of IL-12 in protection against disease has been demonstrated in humans where a defective IL-12 receptor results in an increased susceptibility to mycobacterial infections (de Jong et al., 1998, Altare et al., 1998). IL-18 released by mature DCs, can potentiate IL-12 by inducing expression of IL-12R on NK cells (Novick et al., 2013). Importantly, it has been shown that the ability of DCs to produce IL-12 depends on rapid IFN-γ production by NK cells, resulting in a positive feedback loop involving secretion of IL-12 and IFN-γ from mature DCs and NK cells respectively (Gerosa et al., 2002, Piccioli et al., 2002). NK cells are an important early source of IFN-γ, and NK cell derived IFN-γ contributes to innate resistance to *M. tb* infection of humans (Feng et al., 2006). The development of protective immunity against *M. bovis* infection in cattle is driven by Th1-type immune responses which are characterised by IFN-γ production (Buddle et al., 2005). Interactions between NK cells and DCs *in vivo* have been shown in murine models to mediate the induction of Th1 responses through the secretion of NK cell derived IFN-γ (Martin-Fontecha et al., 2004).

Cell-to-cell contact is required for cross-talk to occur by allowing formation of the immunological synapse for polarised delivery of cytokines and also receptor-ligand interactions. Interactions between CX_3CL1 (fractalkine) and the receptor CX_3CR1 which are expressed by mature DCs and NK cells respectively, aid tethering of both cell populations and this contact stimulates the production of IFN-γ by NK cells (Pallandre et al., 2008). Other receptor ligand interactions which are important in the cross talk between NK cells and DCs are CD40-CD40L and CD28-B7. For example, ligation of CD40 on DCs by CD40L expressed by NK cells can augment the production of IL-12 (Mackey et al., 1998).

Interestingly, NK cells are capable of killing autologous immature DCs and this is proposed to permit NK cell selection of more immunogenic DCs in order to enhance protective immunity. Activated human NK cells kill immature DCs through the NKp30 receptor (Ferlazzo et al., 2002) which is mediated by a subset of CD94/NKG2A+ KIR- NK cells (Della Chiesa et al., 2003). Mature DCs are thought to be resistant to NK cell mediated cytotoxicity due to the up-regulation of MHC class I molecules on their surface (Carbone et al., 1999, Wilson et al., 1999, Ferlazzo et al., 2002, Piccioli et al., 2002). NK cell mediated killing of immature DCs is proposed to be a mechanism by which the immune system selects immunogenic DCs for interactions with T cells.

Initial investigations into bovine innate immune cell interactions in the context of mycobacteria showed that a population of NK-like cells from naïve calves produced IFN-γ after interplay with BCG-infected DCs (Hope et al., 2002b). More recently, interactions between NKp46+ NK cells and *M. bovis*-infected DCs were defined and showed that reciprocal interactions occur, resulting in activation of both NK cells and DCs (Siddiqui and Hope, 2012).

Interactions between NK cells and DCs in the context of BCG vaccination, and the key mechanisms involved in this cross-talk have not been elucidated in cattle. Furthermore, the effect of these interactions on the adaptive immune response is unknown. *In vitro* co-cultures between BCG-infected monocyte-derived DCs and autologous NK cells enriched from peripheral blood were established to study the interaction between these two innate immune cell populations.

The hypotheses being addressed in this chapter are that (1) NK cells require interactions with BCG-infected DCs to become phenotypically and functionally mature; and (2) the interaction between NK cells and DCs, in the context of BCG, drives Th1-type immune responses.

5.2 Results

5.2.1 Purification of CD14+ monocytes

Initial investigations focussed on the effect of the vaccine strain BCG Danish on the phenotype and production of cytokines by monocyte-derived DCs. To generate a population of immature monocyte-derived DCs, CD14+ monocytes were isolated from the peripheral blood of calves using MACS MicroBeads conjugated to mouse anti-human CD14 antibody and cultured for three days with recombinant bovine GM-CSF and IL-4. Prior to culture with GM-CSF and IL-4, the purity of monocytes was assessed by labelling cells with goat anti-mouse IgG conjugated to phycoerythrin (PE). Purities of CD14+ monocytes were consistently >98% (Figure 5.1.1). Following three days of culture with GM-CSF and IL-4 to obtain monocyte-derived DCs, DCs were identified by gating FSC^{high} SSC^{high} cells (Figure 5.1.2) which were negative for the dead cell discriminator, Sytox Blue (Figure 5.1.3).

5.2.2 Infection of DCs with fluorescently labelled BCG

To assess the percentage of DCs that took up BCG bacilli, uptake of bacteria was determined by labelling BCG with FITC. Monocyte-derived DCs from four calves were infected with fluorescently labelled BCG (MOI 5) for 42 hours which is the duration of the co-culture between BCG-infected DCs and NK cells. This MOI was selected after determining the percentage of DCs that took up BCG-FITC and the viability of DCs after infection of DCs with BCG at MOIs of 1, 3 and 5 (as detailed Figure 5.2.1 and 5.2.2). The percentage of BCG-FITC+ cells was determined by flow cytometry. BCG was successfully taken up by immature DCs as illustrated by the percentage uptake of BCG-FITC with 64.5% (48.2-75.8%; SD=14.1) of DCs becoming infected with BCG (Figure 5.2.3).

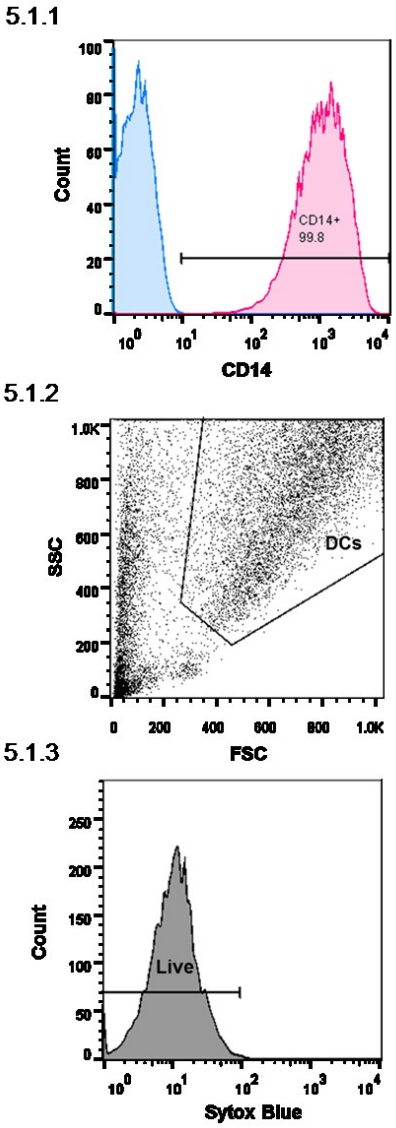


Figure 5.1 Purification of CD14+ monocytes

CD14+ monocytes were positively selected using MACS MicroBeads conjugated to mouse anti-human CD14 antibody and labelled with goat anti-mouse IgG PE antibody to assess purity. A representative FACS plots indicates the purity of CD14+ monocytes from one animal with the red histogram representing cells positive for CD14 (Figure 5.1.1). Gates were set using unstained cells (blue histogram). Purities of CD14+ monocytes were consistently >98%. CD14+ monocytes were cultured for three days with recombinant bovine GM-CSF and IL-4 to obtain monocyte-derived DCs. DCs were identified by gating FSC^{high} SSC^{high} cells (Figure 5.1.2) which were negative for the dead cell discriminator, Sytox Blue (Figure 5.1.3).

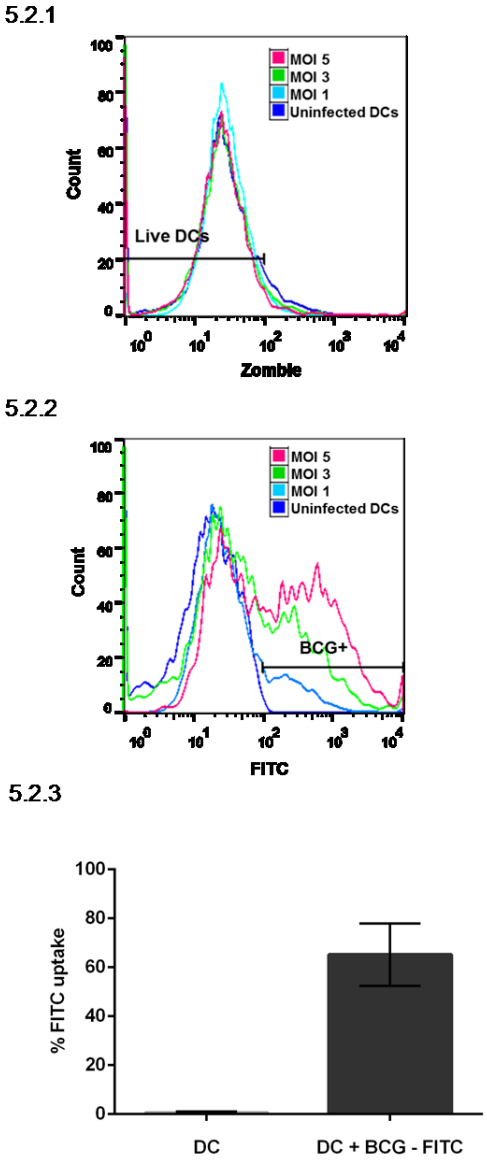


Figure 5.2 Infection of DCs with fluorescently labelled BCG

Monocyte-derived DCs were cultured for three days and infected with FITC-labelled BCG (MOI 1, 3 and 5) for 42 hours. FACS plots from one representative animal (Figure 5.2.1) illustrate viability of DCs after infection with BCG. FACS plots from one representative animal (Figure 5.2.2) the uptake of BCG-FITC by uninfected DCs and BCG-infected DCs at MOI 1, 3 and 5. An MOI of 5 was selected and pooled data from four calves (Figure 5.2.3) illustrate the average percentage uptake of BCG-FITC ± SD by uninfected DCs (lighter bar) and BCG-infected DCs (darker bar).

5.2.3 Expression of MHC and costimulatory molecules by BCG-infected DCs

Following confirmation that immature DCs can uptake and be infected by BCG, the effect of BCG on the phenotype of DCs was investigated by comparing the expression of MHC class II and the costimulatory molecules CD40 and CD80 by uninfected and BCG-infected DCs. Monocyte-derived DCs from four calves were infected with BCG (MOI 5) for 42 hours and the expression of MHC class II, CD40 and CD80 were assessed by flow cytometry (Figure 5.3). Expression of MHC class II ($p<0.001$), CD40 ($p=0.032$) and CD80 ($p=0.044$) were significantly enhanced following infection of DCs with BCG compared with uninfected DCs. Uninfected DCs expressed MHC class I and CD86, however no significant increases were observed in the expression of MHC class I and CD86 by BCG-infected DCs (data not shown). CCR7 expression was not detected by uninfected or BCG-infected DCs (data not shown).

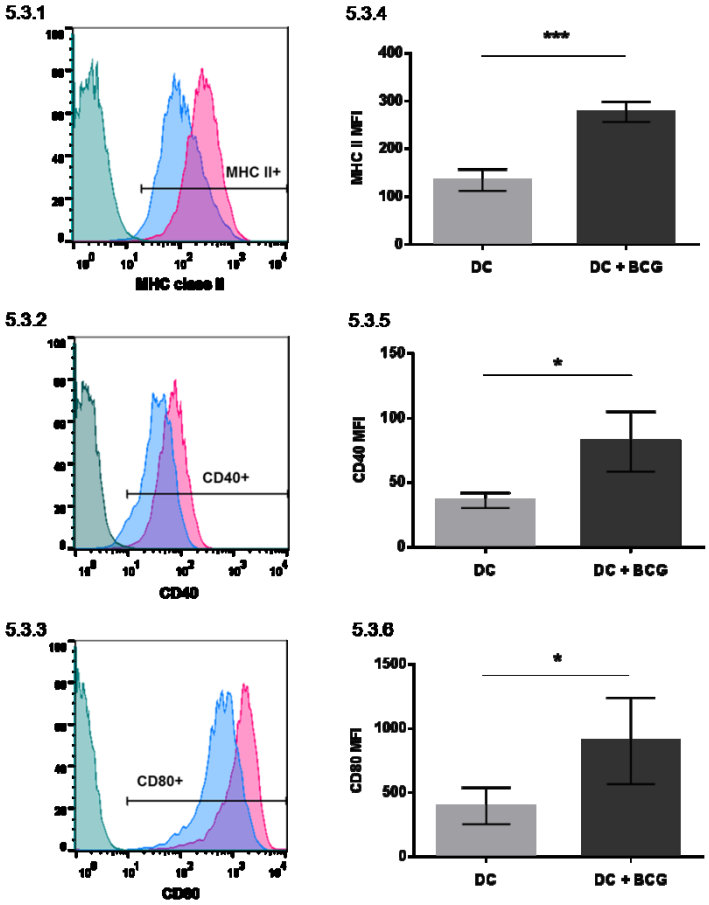


Figure 5.3 Expression of MHC and costimulatory molecules by BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG (MOI 5) for 42 hours. Uninfected and BCG-infected DCs were labelled with mAbs for MHC class II, CD40 and CD80 and analysed by flow cytometry. FACS plots from one representative animal show the expression of MHC class II (Figure 5.3.1), CD40 (Figure 5.3.2) and CD80 (Figure 5.3.3) by uninfected (blue histograms) and BCG-infected DCs (red histograms). Positive cells were identified based on FMO controls (green histograms). Pooled data from four calves indicates the average MFI ± SD of MHC class II (Figure 5.3.4), CD40 (Figure 5.3.5) and CD80 (Figure 5.3.6) expression by uninfected (lighter bars) and BCG-infected DCs (darker bars). Data were normally distributed ($p>0.05$) and significant changes in the expression of MHC class II, CD40 and CD80 by uninfected and BCG-infected DCs were assessed using a 2-sample t-test; $p<0.05$*, $p<0.001$***.

5.2.4 Production of IL-12 by BCG-infected DCs

Protective immunity against *M. bovis* infection in cattle is driven by Th1 polarised immune responses (Buddle et al., 2005), therefore the production of the Th1 polarising cytokine, IL-12 by uninfected and BCG-infected DCs was defined. Monocyte-derived DCs from five calves were infected with BCG (MOI 5) for 42 hours and supernatants were assayed for the presence of IL-12 by ELISA (section 2.15). DCs infected with BCG secreted significantly higher levels of IL-12 ($p=0.023$) compared to uninfected DCs (Figure 5.4).

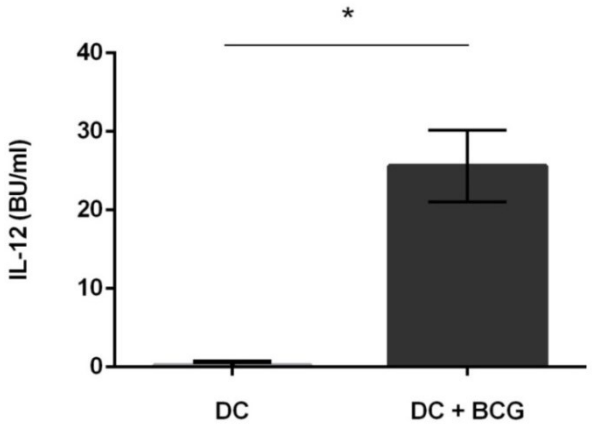


Figure 5.4 Production of IL-12 by BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG (MOI 5) for 42 hours. Supernatants were retrieved from uninfected and BCG-infected DCs and assayed for cytokine production by ELISA (section 2.15). Pooled data from five calves represent the average levels ± SD of IL-12 BU/ml produced by uninfected (lighter bars) and BCG-infected DCs (darker bars). Data were normally distributed ($p>0.05$) and significance between uninfected and BCG-infected DCs was assessed using a 2-sample t-test. $p<0.05$*.

5.2.5 Isolation of bovine peripheral blood NK cells

To explore the interaction between NK cells and DCs in the context of BCG, *in vitro* co-cultures were established between these two populations of innate immune cells and the effect of this interaction on both NK cells and DCs was assessed. NK cells were isolated from peripheral blood using mouse anti-ovine NKp46 mAb (which cross-reacts with bovine) and pan anti-mouse IgG Dynabeads. Following dissociation of the magnetic beads, NK cell purity was confirmed by labelling cells with mouse anti-ovine NKp46 indirectly conjugated to goat anti-mouse IgG PE. The purity of positively selected NK cells was consistently >95% (Figure 5.5).

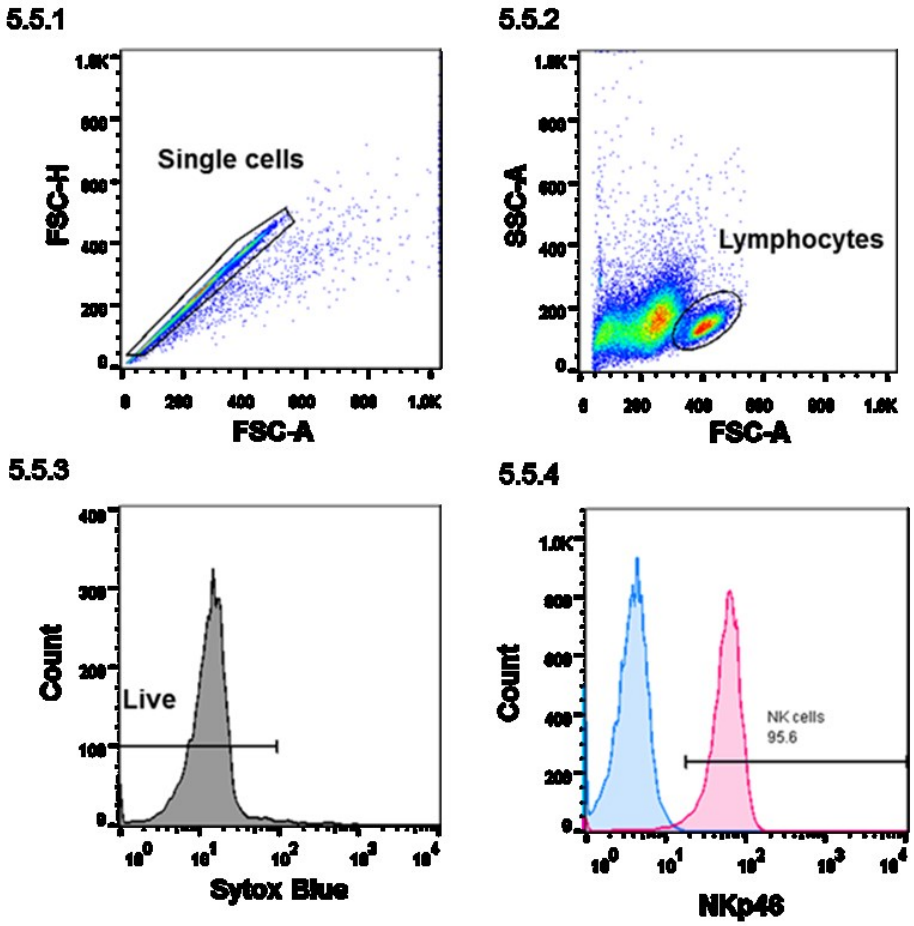


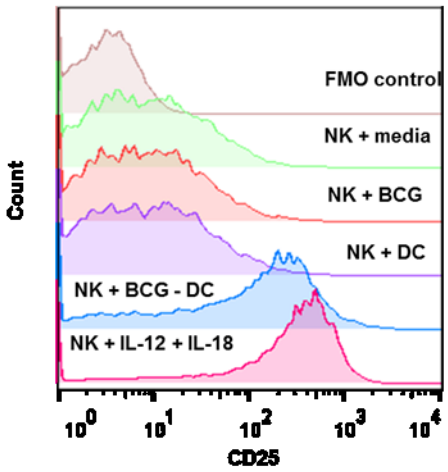
Figure 5.5 Isolation of bovine peripheral blood NK cells

NK cells were isolated by labelling lymphocytes with mouse anti-ovine NKp46 followed by positive selection using pan-mouse IgG Dynabeads. NK cell purity was assessed by labelling cells with mouse anti-ovine NKp46 indirectly conjugated to goat anti-mouse IgG PE. Representative FACS plots illustrate the gating strategy used to identify bovine NK cells. Single cells were gated (Figure 5.5.1), followed by lymphocytes (Figure 5.5.2) which were negative for the dead cell discriminator, Sytox Blue (Figure 5.5.3). Figure 5.5.4 indicates the purity of NKp46+ NK cells (red histogram). Gates were set using unstained cells (blue histogram). Purities of positively selected NK cells were consistently >95%.

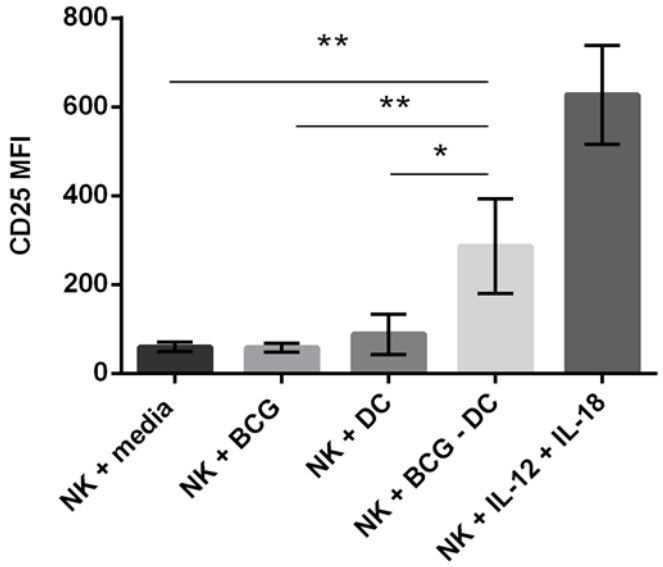
5.2.6 Activation of NK cells following co-culture with BCG-infected DCs

To assess the activation status of NK cells after *in vitro* co-culture with BCG-infected DCs, the expression of CD25 was examined using flow cytometry. Monocyte-derived DCs from five calves were infected with BCG (MOI 5) for 24 hours and cultured for a further 18 hours with autologous NKp46+ NK cells enriched from peripheral blood. NK cells cultured with media, BCG, uninfected DCs or with recombinant bovine IL-12 and recombinant human IL-18 served as controls. CD25 expression, as represented by the MFI, was low when NK cells were cultured with media, with BCG and with uninfected DCs (Figure 5.6.2). Subsequent to co-culture with BCG-infected DCs, NK cell expression of CD25 was significantly increased compared with when NK cells were cultured with media ($p=0.009$), with BCG ($p=0.009$) and with uninfected DCs ($p=0.012$). NK cells supplemented with IL-12 and IL-18 (positive control), had a high expression of CD25 (Figure 5.6.2). In addition to enhanced CD25 expression (as assessed by MFI) a similar significant increase in the percentage of CD25+ NK cells present after culture with BCG-infected DCs was evident compared with the percentage of CD25+ NK cells present after culture of NK cells with media ($p=0.005$), BCG ($p=0.004$) and uninfected DCs ($p=0.034$) (Figure 5.6.3). Expression levels of NKp30 by NK cells after co-culture with BCG-infected DCs were examined and were undetectable across all conditions (data not shown).

5.6.1


FMO control
NK + media
NK + BCG
NK + DC
NK + BCG - DC
NK + IL-12 + IL-18
Count
CD25

5.6.2


CD25 MFI
800
600
400
200
0
**
**
*
NK + media
NK + BCG
NK + DC
NK + BCG - DC
NK + IL-12 + IL-18

5.6.3

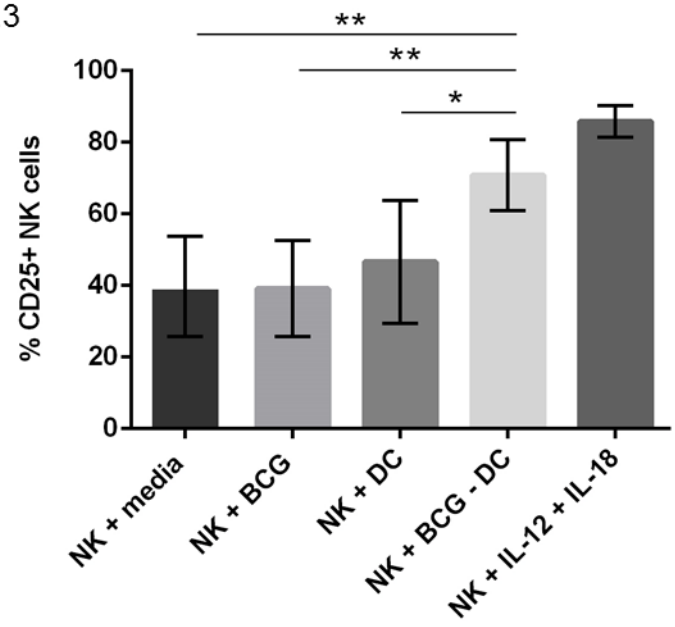

% CD25+ NK cells
100
80
60
40
20
0
**
**
*
NK + media
NK + BCG
NK + DC
NK + BCG - DC
NK + IL-12 + IL-18

Figure 5.6 Activation of NK cells following co-culture with BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG (MOI 5) for 24 hours. NKp46+ cells were enriched from peripheral blood and cultured with BCG-infected DCs at a ratio of 5 NK cells per DC (section 2.7). NK cells cultured with media, BCG, uninfected DCs or with IL-12 and IL-18 served as controls. After 18 hours of co-culture, cells were labelled with mAbs for NKp46 and CD25 and analysed by flow cytometry. FACS plots from one representative animal illustrate the expression of CD25 by NKp46+ NK cells after culture with media, BCG, uninfected DCs, BCG-infected DCs or with IL-12 and IL-18 (Figure 5.6.1). Positive cells were identified based on FMO controls. Pooled data from five calves illustrate the average MFI ± SD of CD25 expression by NK cells (Figure 5.6.2). Pooled data from five calves displays the average percentage of CD25+ NK cells ± SD (Figure 5.6.3). Data were normally distributed ($p>0.05$) and significance between co-culture conditions was assessed using 2-sample t-tests. $p<0.05$*, $p<0.01$**.

5.2.7 Activation of NK cell subsets following co-culture with BCG-infected DCs

To determine if the observed activation (CD25 expression) of NK cells in response to culture with BCG-infected DCs (Figure 5.2.6) was attributed to a particular subset of NK cells, multicolour flow cytometry was used to assess the expression of CD25 by CD2+ and CD2- subsets of NK cells. Expression was compared following culture of NK cells with media, BCG, uninfected DCs, BCG-infected DCs or when stimulated with IL-12 and IL-18. CD25 expression did not significantly differ between CD2+ and CD2- subsets when NK cells were cultured with media, BCG, uninfected DCs or with IL-12 and IL-18. However, after culture with BCG-infected DCs, CD2- NK cells had a significantly higher expression of CD25 than CD2+ NK cells ($p=0.017$) (Figure 5.7).

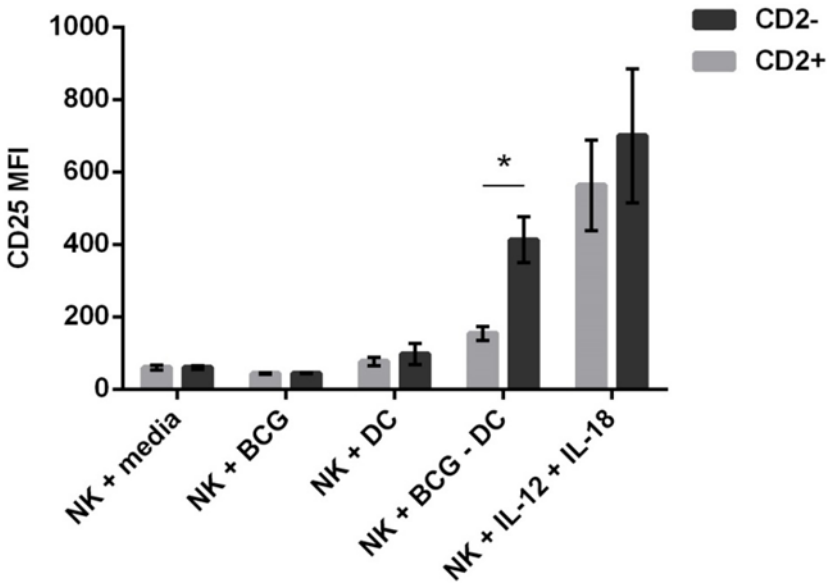


Figure 5.7 Activation of NK cell subsets following co-culture with BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG (MOI 5) for 24 hours. NKp46+ cells were enriched from peripheral blood and cultured with infected DCs at a ratio of 5 NK cells per DC (section 2.8). NK cells cultured with media, BCG, uninfected DCs or with IL-12 and IL-18 served as controls. After 18 hours of co-culture, cells were labelled with mAbs for NKp46, CD2 and CD25 and analysed by flow cytometry. Positive cells were identified based on FMO controls. Pooled data from five calves indicate the average MFI ± SD of CD25 expression by CD2+ (lighter bars) and CD2- (darker bars) NK cells. Data were normally distributed ($p>0.05$) and significance between the expression of CD25 by CD2+ and CD2- NK cells was assessed using a 2-sample t-test. $p<0.05$*.

5.2.8 Activation of NKp46+ CD3+ lymphocytes following co-culture with BCG-infected DCs

A population of NKp46+ CD3+ lymphocytes which have shared attributes of both bovine NK cells and T cells has recently been described (Connelley et al., 2014). As the enrichment technique used to isolate bovine NK cells from peripheral blood involves positively selecting the total NKp46+ population, the observed activation of NK cells as a result of *in vitro* co-culture with BCG-infected DCs (Figure 5.2.6) may be due (at least in part) to activation of these double positive cells and not solely to NKp46+ CD3- NK cells. To address whether NKp46+ CD3+ cells were activated after co-culture with BCG-infected DCs, the expression of CD25 was analysed on NKp46+ CD3+ and NKp46+ CD3- cells. Monocyte-derived DCs from three calves were infected with BCG for 24 hours and then cultured for 18 hours with autologous NKp46+ NK cells enriched from peripheral blood. The activation status of NKp46+ CD3+ and NKp46+ CD3- cells was determined by assessing the percentage of CD3+ or CD3- cells within the total NKp46+ CD25+ population. CD3- cells accounted for a significantly higher ($p<0.001$) percentage of the NKp46+ CD25+ population in comparison to CD3+ cells, with 98.4% (97.7-98.9; SD=0.51) of the NKp46+ CD25+ population being CD3- (Figure 5.8.4).

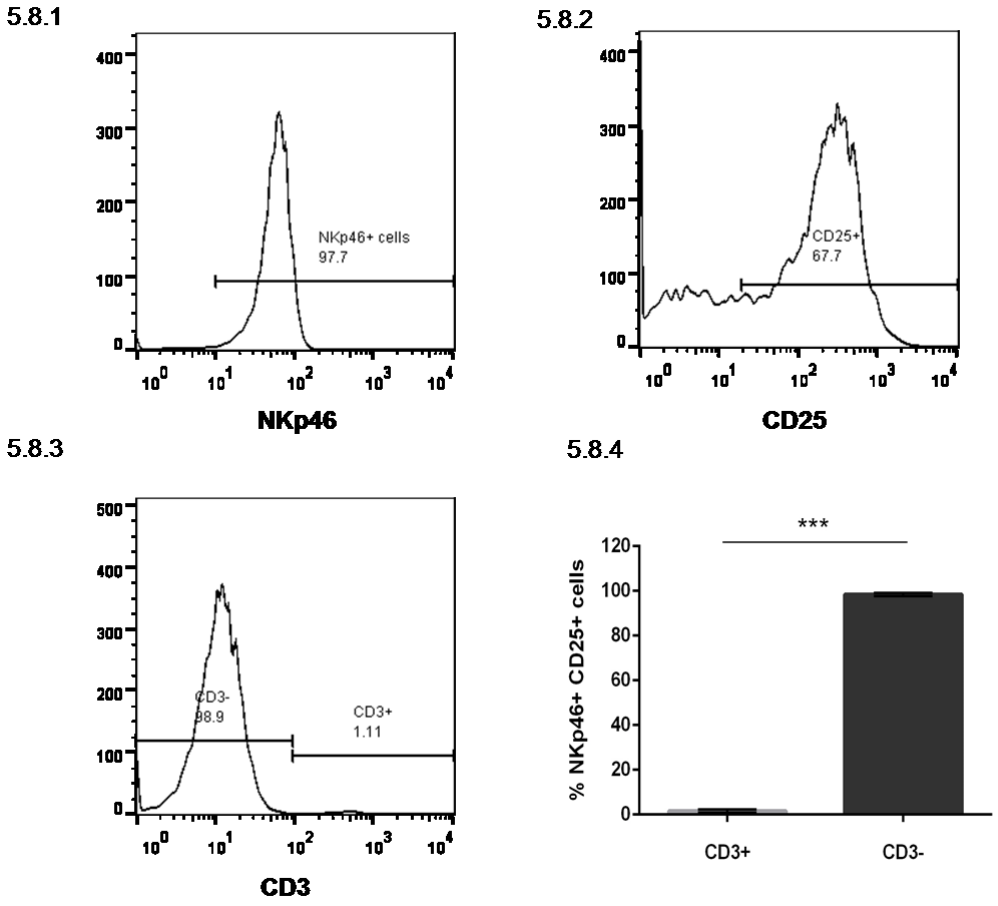


Figure 5.8 Activation of NKp46+ CD3+ lymphocytes following co-culture with BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG for 24 hours. NKp46+ cells were enriched from peripheral blood and cultured with infected DCs at a ratio of 5 NK cells per DC (section 2.7). After 18 hours of co-culture, cells were labelled with mAbs for CD3, NKp46 and CD25 and analysed by flow cytometry. FACS plots from one representative animal denotes the gating strategy used to assess the percentage of CD3+ and CD3- cells within the total gated NKp46+ CD25+ cells. Gates were set using FMO controls. NKp46+ NK cells were selected (Figure 5.8.1) followed by CD25+ NK cells (Figure 5.8.2) and then CD3+ and CD3- cells were gated (Figure 5.8.3). Pooled data from three calves illustrates the percentage of CD3+ (lighter bar) and CD3- (darker bar) cells ± SD within the NKp46+ CD25 population (Figure 5.8.4). Data were normally distributed ($p>0.05$) and significance between CD3+ and CD3- cells was assessed using a 2-sample t-test; $p<0.001$***.

5.2.9 NK cell production of IFN-γ after co-culture with BCG-infected DCs

In addition to the activation status of NK cells, the effector function of NK cells following co-culture with BCG-infected DCs was investigated by determining NK cell production of IFN-γ. Supernatants were retrieved from co-culture experiments (section 2.7) and assayed for IFN-γ by ELISA (section 2.15). IFN-γ production was low when NK cells were cultured with media, with BCG or with uninfected DCs. Following co-culture with BCG-infected DCs, NK cell production of IFN-γ was significantly augmented compared with NK cells cultured with media ($p=0.020$), NK cells cultured with BCG ($p=0.021$) and NK cells cultured with uninfected DCs ($p=0.021$). NK cells supplemented with IL-12 and IL-18 (positive control) produced high levels of IFN-γ (Figure 5.9).

5.2.10 Effect of NK cell-DC co-culture on cytokine production by DCs

It is well established in the literature that interactions between NK cells and DCs are reciprocal, thus the production of DC derived IL-12 was measured after co-culture with NK cells. Supernatants were retrieved from co-culture experiments (section 2.7) and assayed for IL-12 by ELISA (section 2.15). As illustrated previously, DCs secreted elevated levels of IL-12 upon infection with BCG compared with the low levels produced by uninfected DCs (Figure 5.2.4). Similar to DCs cultured with media, uninfected DCs cultured with NK cells produced low levels of IL-12. Addition of NK cells to BCG-infected DCs did not significantly increase the level of IL-12 produced by BCG-infected DCs alone (Figure 5.10).

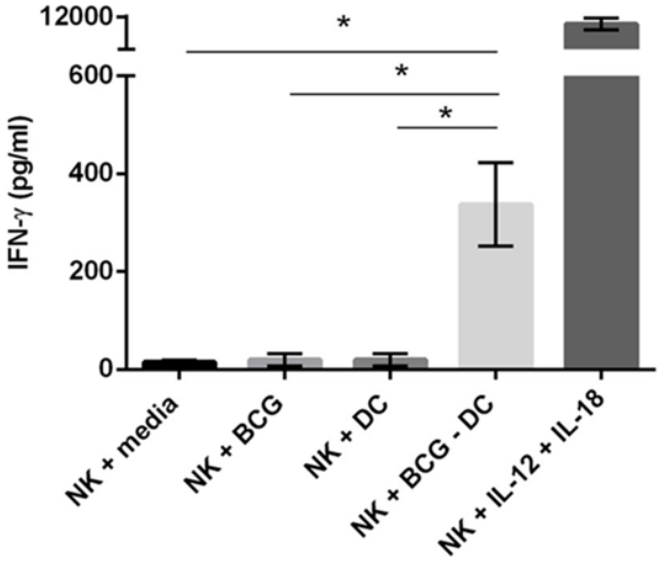


Figure 5.9 NK cell production of IFN-γ after co-culture with BCG-infected DCs

Monocyte-derived DCs were cultured for three days and infected with BCG for 24 hours. NK cells were enriched from peripheral blood and cultured with infected DCs at a ratio of 5 NK cells per DC (section 2.7). NK cells cultured with media, BCG alone, uninfected DCs or with IL-12 and IL-18 served as controls. After 18 hours of co-culture, supernatants were assayed for IFN-γ production by ELISA (section 2.15). Pooled data from five calves represents the average levels ± SD of IFN-γ (pg/ml) produced. Data were normally distributed ($p>0.05$) and significance between co-culture conditions was assessed using 2-sample t-tests. $p<0.05$*.

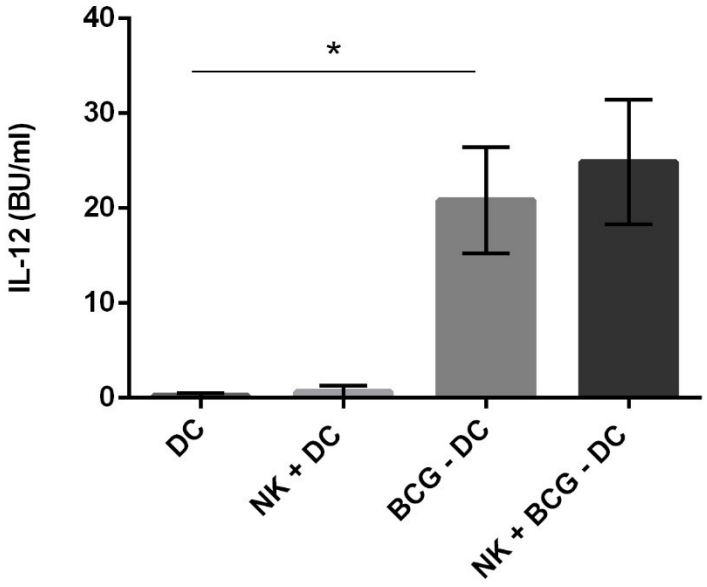


Figure 5.10 Effect of NK cell-DC co-culture on DCs

Monocyte-derived DCs were cultured for three days and infected with BCG for 24 hours. NK cells were enriched from peripheral blood and cultured with infected DCs at a ratio of 5 NK cells per DC (section 2.7). DCs cultured with media, BCG or NK cells served as controls. After 18 hours of co-culture, supernatants were assayed for production of IL-12 by ELISA (section 2.15). Pooled data from five calves represents the average levels ± SD of IL-12 (BU/ml). Data were normally distributed ($p>0.05$) and significance between co-culture conditions was assessed using a 2-sample t-test. $p<0.05$*.

5.2.11 Activation of NK cells following co-culture with Zylexis™-exposed DCs

In parallel with BCG, Zylexis™, an inactivated equine Parapox Ovis Virus Immunomodulator, was tested within *in vitro* co-cultures of NK cells and DCs. Monocyte-derived DCs from one animal were exposed to Zylexis™ at a MOI of 1 or with BCG at an MOI of 5 for 24 hours and cultured for a further 18 hours with autologous NKp46+ NK cells enriched from peripheral blood. An optimal MOI of 1 for the exposure of DCs to Zylexis™ was decided by determining cell viability after exposure of DCs with Zylexis™ at MOIs of 1, 5 and 10. NK cells cultured with media, Zylexis™, BCG, uninfected DCs or supplemented with IL-12 and IL-18 served as controls. NK cell activation, as defined by the expression of CD25, was analysed by flow cytometry (Figure 5.11.1). CD25 expression was low when NK cells were cultured with media, Zylexis™, BCG, uninfected DCs and Zylexis™-infected DCs. Similar to Figure 5.2.6, NK cell activation was augmented after culture with BCG-infected DCs or when supplemented with IL-12 and IL-18. To assess if culture of NK cells with Zylexis™-exposed DCs altered the expression of CD25 by CD2+ or CD2- subsets, CD25 expression by CD2+ and CD2- NK cells was compared following culture with media, Zylexis™, BCG, uninfected DCs, Zylexis™-exposed DCs, BCG-infected DCs or with IL-12 and IL-18 (Figure 5.11.2). No difference in the expression of CD25 by CD2+ and CD2- NK cells was evident after culture with Zylexis™-exposed DCs. As illustrated previously (Figure 5.7), CD2- NK cells have an increased expression of CD25 after co-culture with BCG-infected DCs.

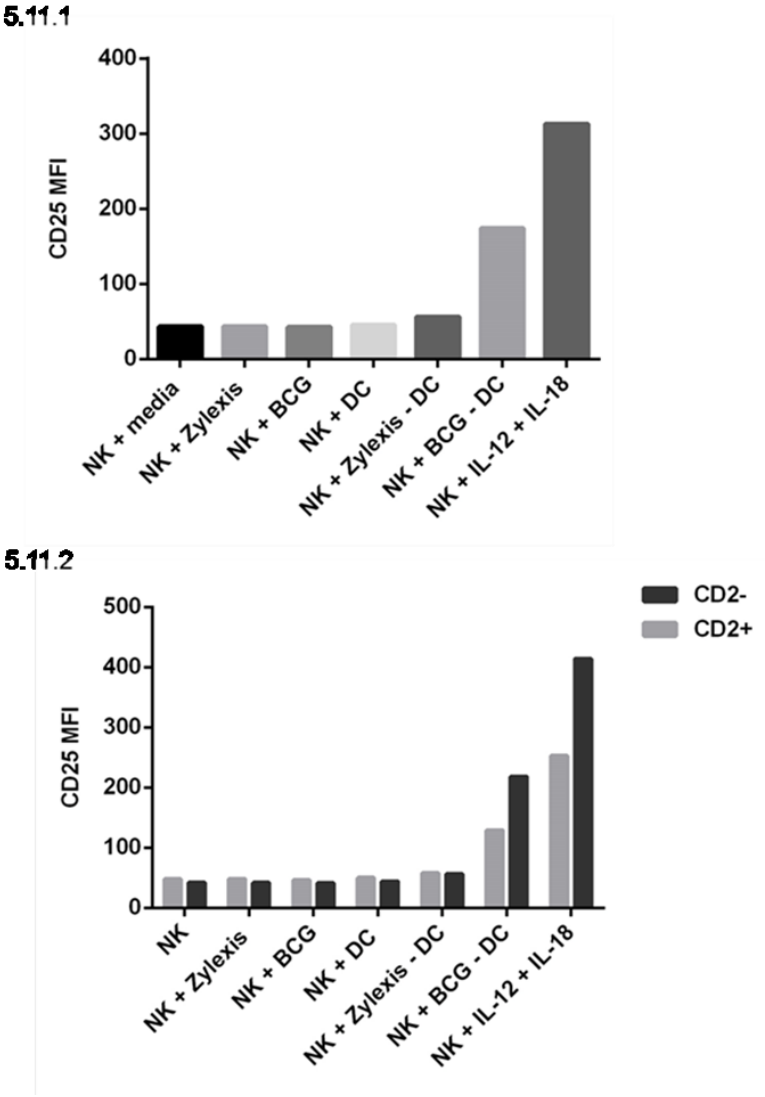


Figure 5.11 Activation of NK cells following co-culture with Zylexis™-exposed DCs

Monocyte-derived DCs were cultured for three days and exposed to Zylexis™ (MOI 1) or infected with BCG (MOI 5) for 24 hours. NKp46+ cells were enriched from peripheral blood and cultured with Zylexis™-exposed or BCG-infected DCs at a ratio of 5 NK cells per DC (section 2.8). NK cells cultured with media, Zylexis™, BCG, uninfected DCs or with IL-12 and IL-18 served as controls. After 18 hours of co-culture, cells were labelled with mAbs for NKp46, CD2 and CD25 and analysed by flow cytometry. Positive cells were identified based on FMO controls. Data from one animal illustrates the MFI of CD25 expression by NKp46+ NK cells (Figure 5.11.1) and the expression of CD25 by CD2+ (lighter bars) and CD2- NK cells (darker bars) (Figure 5.11.2).

5.2.12 IFN-γ production by NK cells following co-culture with Zylexis™-exposed DCs

Zylexis™ had no effect on the activation of NK cells (Figure 5.11.1) however Zylexis™ may have induced IFN-γ production by NK cells and therefore be having a functional effect. To assess if co-culture with Zylexis™-exposed DCs affected the effector function of NK cells, the production of IFN-γ was assessed. Supernatants were retrieved from co-culture experiments (section 2.8) and NK cell production of IFN-γ was determined by ELISA (section 2.15). Similar to the low level of IFN-γ produced after culture of NK cells with media, Zylexis™, BCG and uninfected DCs, IFN-γ production was not enhanced following NK cell culture with Zylexis™-exposed DCs (Figure 5.12). As previously shown in Figure 5.2.9, co-culture of NK cells with BCG-infected DCs or stimulation with IL-12 and IL-18 resulted in enhanced production of IFN-γ.

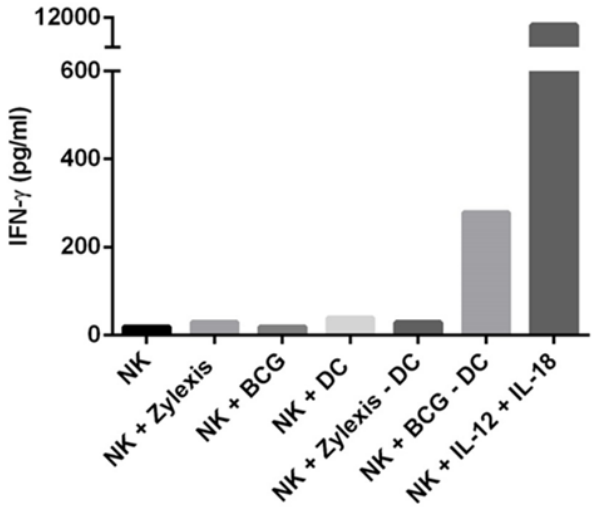


Figure 5.12 IFN-γ production by NK cells following co-culture with Zylexis™-exposed DCs

Monocyte-derived DCs were cultured for three days and were exposed to Zylexis™ (MOI 1) or infected with BCG (MOI 5) for 24 hours. NKp46+ cells were enriched from peripheral blood and cultured with Zylexis™-exposed or BCG-infected DCs at a ratio of 5 NK cells per DC (section 2.8). NK cells cultured with media, Zylexis™, BCG, uninfected DC or with IL-12 and IL-18 served as controls. After 18 hours of co-culture, the amount of IFN-γ present in the supernatant was measured by ELISA (section 2.15). Data from one animal indicates the production of IFN-γ (pg/ml) by NK cells.

5.3 Discussion

To determine the effect of BCG on NK cell-DC interactions, *in vitro* co-cultures between these two innate immune cell populations were established. Prior to assessment of the effect of culture with BCG-infected DCs on the activation and effector function of NK cells, the influence of the vaccine strain BCG Danish on DC phenotype and cytokine production was initially determined. CD14+ monocytes cultured with recombinant bovine GM-CSF and IL-4 for 3 days represent a population of immature monocyte-derived DCs (Werling et al., 1999). In line with published data by Werling *et al*, immature, uninfected DCs in this study expressed MHC class II, CD40 and CD80 at low levels which were increased upon pathogenic stimuli (Figure 5.3). Immature monocyte-derived DCs ingested BCG as illustrated by the uptake of fluorescently labelled bacteria (Figure 5.2.3). Receptors involved in the uptake of mycobacteria by human and murine DCs include dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Tailleux et al., 2003), TLR2 (Means et al., 1999), TLR4 (Tsuji et al., 2000) and TLR9 (von Meyenn et al., 2006). Similarly, a role for DC-SIGN in the uptake of BCG by bovine monocyte-derived DCs was confirmed when it was demonstrated that blocking DC-SIGN with a polyclonal antibody reduced binding of GFP-labelled BCG (Yamakawa et al., 2008). Furthermore, bovine monocyte-derived DCs express surface TLR2 which could be a potential route for uptake of BCG in cattle.

Upon antigen uptake, immature DCs undergo maturation characterised by the upregulation of MHC class I, MHC class II and costimulatory molecules, including CD40, CD80 and CD86 (Steinman, 1991). Following uptake of BCG Danish by bovine DCs, DCs significantly increased their expression of MHC class II, CD40 and CD80 reflecting maturation of DCs in response to BCG (Figure 5.3). This is in line with published data whereby DCs infected with BCG Pasteur showed an increased expression of MHC class II, CD40 and CD80 (Hope et al., 2004). Taken together, this provides evidence that bovine monocyte-derived DCs undergo maturation in response to BCG Pasteur and to the human vaccine strain BCG Danish. Non-significant increases were noted in the expression of MHC class I and CD86 by BCG-infected DCs compared with uninfected DCs (data not shown). Maturing DCs also express

chemokine receptors, for example, CCR7 which permits migration of DCs carrying antigen from the periphery to the draining LNs (Moretta, 2002). This allows entry of mature DCs, which are licensed to effectively stimulate naïve T cells, into the LNs. However, expression of CCR7 was not detected by uninfected or BCG-infected bovine DCs (data not shown).

In parallel with phenotypic maturation in response to antigen uptake, DCs also secrete cytokines which direct appropriate adaptive immune responses, and therefore link the innate and the adaptive immune response. Naïve CD4+ T cells can differentiate into various subsets of CD4+ T helper cells which is dictated in part by the cytokine milieu present at the time of differentiation (Zhou et al., 2009). For example, the presence of IL-12 and IFN-γ in the local environment results in the development of a Th1 immune response whereas the presence of IL-4 directs a Th2 immune response. Given that that Th1 dominant response is likely to be most effective against *M. bovis* infection, the production of IL-12 by uninfected and BCG-infected DCs was quantified. BCG-infected DCs produced significant levels of IL-12 after infection with BCG (Figure 5.4) indicating that BCG-infected DCs could contribute significantly to the induction of a CD4+ Th1 immune response. Bovine DCs have been shown previously to secrete IL-12 after infection with *M. bovis* and the Pasteur strain of BCG (Hope et al., 2004). The results presented in Figure 5.4 demonstrate that DCs can also produce IL-12 when stimulated with the vaccine strain of BCG. Conversely, BCG-infected macrophages have been reported to produce very little IL-12 (Hope et al., 2004), suggesting that macrophages are unlikely to be the drivers of Th1 responses to BCG.

After establishing that DCs mature in response to infection with BCG (Figure 5.3) and produced the Th1 polarising cytokine IL-12 (Figure 5.4), the effect of BCG-infected DCs on NK cell activation was investigated by assessing NK cell expression of CD25. To do this, *in vitro* co-cultures were established between monocyte-derived DCs infected with BCG and autologous peripheral blood derived NK cells at an optimal ratio of 5 NK cells per DC (Siddiqui and Hope, 2012). CD25 expression was significantly augmented when NK cells were cultured with BCG-infected DCs, reflecting activation of NK cells in response to co-culture with DCs in the context of BCG (Figure 5.6). Previous work has shown that bovine NK cells are optimally

stimulated with IL-12 and IL-18 (Siddiqui and Hope, 2012), therefore NK cells supplemented with IL-12 and IL-18 were used as a positive control. NK cells stimulated in this manner were highly activated. CD25 is the α chain of the IL-2R and together with the IL-2Rβ and γ chains allows IL-2 signalling through the IL-2R, therefore data presented in Figure 5.6 suggests NK cells are more responsive to IL-2 following co-culture with BCG-infected DCs. It was also demonstrated in Figure 5.8.4 that activation of NK cells after co-culture with BCG-infected DCs was specific to NKp46+ CD3- NK cells and that a recently characterised subset of lymphocytes that co express NKp46 and CD3 were not activated by co-culture with BCG-infected DCs (Connelley et al., 2014).

The observed activation of NK cells after *in vitro* co-culture with BCG-infected DCs (Figure 5.6) appears to be due to preferential activation of the CD2- subset of NK cells illustrated by a significantly higher CD25 expression by CD2- NK cells compared with CD2+ NK cells (Figure 5.7). Interestingly, in the positive control whereby NK cells were stimulated with IL-12 and IL-18, there was not a significant difference between the expression of CD25 by the two subsets, indicating that the increased activation of CD2- NK cells after co-culture with BCG-infected DCs was specific to these conditions. This preferential activation of bovine CD2- NK cells was also apparent when NK cells were cultured with *M. bovis*-infected DCs (Siddiqui and Hope, 2012) showing that CD2- NK cells are preferntially activated when NK cells are cultured with DCs *in vitro* in the context of both BCG and *M. bovis*.

NKp30 is a key molecule involved in NK cell killing of immature DCs in humans (Ferlazzo et al., 2002) therefore expression of this receptor by NK cells was assessed after co-culture with BCG-infected DCs. Using a mAb to bovine NKp30, NKp30 expression was not detected on NK cells across all culture conditions (data not shown). In other experiments, NKp30 was upregulated by peripheral blood NK cells after overnight culture with recombinant bovine IL-2 (data not shown). It is proposed that NK cells activated by BCG-infected DCs kill immature DCs to allow the immune system to select the most immunogenic DCs (Siddiqui and Hope, 2012). Activated human NK cells which have been exposed to *M. bovis*-infected DCs acquire the ability

to kill immature DCs whereas DCs which upregulate HLA class I molecules were resistant to NK cell mediated killing (Ferlazzo et al., 2003). Similarly, bovine NK cells increased their expression of perforin and capacity to kill P815 target cells after interactions with bovine *M. bovis*-infected DCs (Siddiqui and Hope, 2012). Further work is required to address this hypothesis in cattle and the key molecules involved in this process.

Production of cytokines, primarily IFN-γ, is a key functional property of NK cells and in parallel with IL-12, early production of IFN-γ drives optimal Th1 polarised immune responses. Therefore, supernatants from co-culture experiments were assayed for the presence of NK cell-derived IFN-γ. IFN-γ production was significantly augmented when NK cells were co-cultured with BCG-infected DCs (Figure 5.9), demonstrating that production of IFN-γ by NK cells after co-culture with BCG-infected DCs has the potential to prime Th1-type immunity. It is not clear if the concentration of IFN-γ produced by NK cells after *in vitro* co-culture with BCG-infected DCs is biologically relevant. To test this, the ability of macrophages to kill BCG after stimulation with titrated IFN-γ could be tested *in vitro*. Murine NK cells polarise Th1 immune responses through interactions with DCs (Martin-Fontecha et al., 2004) and enhanced secretion of IFN-γ by NK cells was also noted after reciprocal interactions with *M. tb*-infected DCs (Gerosa et al., 2002). Within the present study, the subset of NK cells responsible for the increased IFN-γ production following co-culture with BCG-infected DCs was not defined. However, bovine CD2- NK cells have an increased capacity to produce IFN-γ compared with CD2+ NK cells (Boysen et al., 2006). Furthermore, CD2- NK cells are the subset responsible for production of IFN-γ after culture with *M. bovis*-infected DCs (Siddiqui and Hope, 2012). Therefore, we hypothesise that CD2- NK cells are the major IFN-γ producers following co-culture with BCG-infected DCs. Studies in humans have shown that BCG can bind directly to NK cells through NK cell expression of TLR2 and NKp44 and induce NK cell production of IFN-γ. Data presented here shows no effect of BCG alone on the production of IFN-γ by NK cells (Figure 5.9), or on the activation of NK cells (Figure 5.6). Despite TLR2 being transcribed by bovine NK cells (Nazneen Siddiqui, PhD thesis, Imperial College, 2011), expression of TLR2 was not detected on the surface of NK cells (data not

shown). Furthermore, NKp44 is a pseudogene in cattle (Hammond, 2012) therefore combined with the lack of surface TLR2, may explain why there was no direct recognition of BCG by bovine NK cells in this study.

Data presented herein demonstrates that BCG-infected DCs provide signals to NK cells which induce phenotypical and functional changes; however NK cells are also thought to modulate the response of DCs in a reciprocal interaction. Thus, the effect of NK cells on the production of IL-12 by BCG-infected DCs was assessed. The addition of NK cells increased the amount of IL-12 produced by BCG-infected DCs alone; although this was not significant (Figure 5.10). NK cells express receptors for IL-12 (Vitale et al., 2002) and as the supernatants were retrieved 18 hours after co-culture with BCG-infected DCs, it is possible that the presence of NK cells enhanced the production of IL-12 by BCG-infected DCs through the release of IFN-γ, and this IL-12 was subsequently utilised by NK cells expressing IL-12R. In mice, DCs that have been in contact with NK cells were shown to increase secretion of IL-12 (Van Elssen et al., 2010) and in cattle, *M. bovis*-infected DCs showed enhanced expression of MHC class II after interactions with autologous peripheral blood NK cells (Siddiqui and Hope, 2012). Hence, NK cells may influence the adaptive immune response by inducing effective APC. As a result of data presented thus far, the proposed *in vitro* interaction between bovine NK cells and DCs in the context of BCG is illustrated in Figure 5.13.

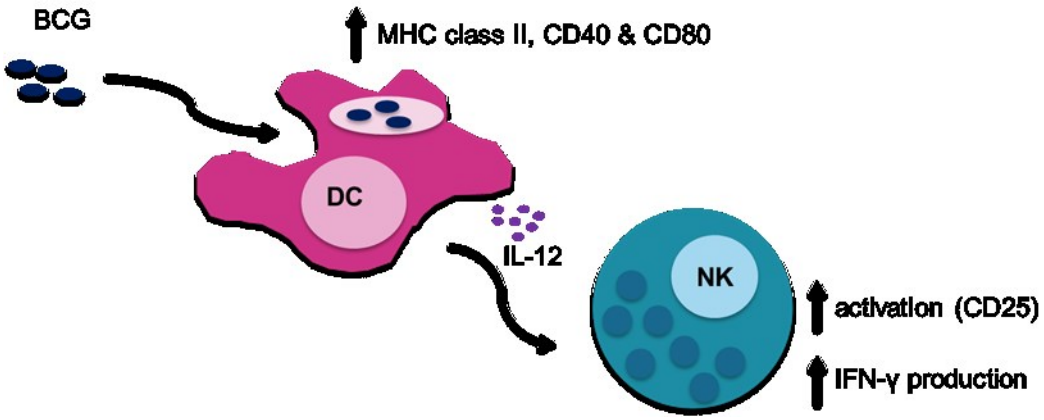


Figure 5.13. The proposed interaction between bovine BCG-infected DCs and NK cells *in vitro*.

Immature monocyte-derived DCs take up BCG resulting in increased expression of MHC class II and the key costimulatory molecules, CD40 and CD80. BCG-infected DCs also produce elevated levels of the Th1 polarising cytokine IL-12. As a result of interactions with BCG-infected DCs, autologous CD2- NK cells increase their expression of the activation marker CD25 and functional capacity, defined by NK cell production of IFN-γ. Together this interaction may contribute to the development of a Th1 biased immune response through the secretion of IL-12 and IFN-γ by BCG-infected DCs and NK cells respectively.

In conjunction with Zoetis, Zylexis™ was tested in co-cultures of NK cells and DCs to determine if Zylexis™ could act as an immunomodulator within this system. Adjuvants such as R848 (TLR7 agonist) recruited NK cells to antigen stimulated LNs of mice, as effectively as LPS-matured DCs, where they polarised Th1 immune responses through the secretion of IFN-γ (Martin-Fontecha et al., 2004). Co-cultures were set up as described in section 2.7 and also included NK cells cultured with Zylexis™ and NK cells cultured with Zylexis™-exposed DCs. Zylexis™ co-cultures were set up in parallel with BCG co-cultures to allow direct comparison between the two agents. Zylexis™-exposed DCs from three calves showed no significant differences in the expression of MHC class II, CD40, CD80 or the production of IL-12 compared with uninfected DCs (data not shown). Subsequently, after co-culture of NK cells with Zylexis™-exposed DCs, there were no significant differences in the expression of CD25 (Figure 5.11.1) or production of IFN-γ (Figure 5.12) by NK cells. However, after co-culture with BCG-infected DCs, NK cell activation and effector function was significantly enhanced as previously illustrated (Figures 5.6 and 5.9). Since Zylexis™ did not induce maturation of DCs; it is perhaps not surprising that culture of NK cells with Zylexis™-exposed DCs had no effect on NK cell activation or function. It was possible that Zylexis™ could act directly on NK cells inducing CD25 expression and IFN-γ production without the requirement of accessory cells such as DCs. Nevertheless, similar to BCG, Zylexis™ alone had no effect on NK cell activation (Figure 5.11) or function (Figure 5.12). It should be noted that the influence of Zylexis™ on DC phenotype and production of IL-12 was demonstrated in three calves, however co-cultures between Zylexis™-infected DCs and autologous NK cells were investigated using DCs and NK cells derived from one animal and therefore may not be fully representative. Further work is required to decipher the effect of such adjuvants on NK cells.

The mechanisms underlying the interactions between bovine NK cells and DCs in the context of BCG have not yet been elucidated. Both soluble factors and contact-dependent receptor ligand interactions are thought to be important. Since uninfected DCs (which have a low expression of MHC class II, CD40 and CD80, and very low levels of IL-12) did not stimulate NK cell activation (Figure 5.6) or production of IFN-

γ (Figure 5.9), we hypothesised that IL-12, CD40 and CD80 were likely to be key molecules involved in the cross-talk between NK cells and DCs. Preliminary experiments utilising an IL-12 neutralising antibody CC326, (Hope et al., 2002a) and CD40 and CD80 blocking antibodies provided some evidence for the involvement of these molecules during the NK cell-DC interaction in the context of BCG, however these experiments require further optimisation. Despite being unable to definitively show that these molecules were involved in the interactions between NK cells and BCG-infected DCs, we were able to demonstrate that exposure of DCs to Zylexis™ did not increase the expression of MHC class II, CD40 or CD80, or the production of IL-12 and subsequently, Zylexis™-exposed DCs were not equipped to induce NK cell activation (Figure 5.11) or cytokine production (Figure 5.12). Overall, IL-12, CD40 and CD80 represent suitable candidates for some of the key molecules involved in the crosstalk between NK cells and BCG-infected DCs.

To conclude, data presented in this Chapter has demonstrated that bovine NK cells require interactions with BCG-infected DCs for optimal activation and production of IFN-γ *in vitro*, therefore proving the first hypothesis stated at the beginning of this Chapter. Furthermore, CD2- NK cells preferentially interact with DCs in the context of BCG highlighted by their increased activation compared with their CD2+ counterparts. Through the secretion of IFN-γ and IL-12 by NK cells and BCG-infected DCs respectively, this interaction generated a cytokine milieu which is conducive to the development of Th1 biased CD4+ and CD8+ T cell response, thus proving the second part of the hypothesis. Further studies are required to determine if the interaction between NK cells and BCG- or *M. bovis*-infected DCs primes $CD8^+$ T cells and Th1 polarised $CD4^+$ T cells.

The species and subspecies of the *M. tb* complex infect a wide range of mammalian hosts, with *M. tb* and *M. bovis* being the causative agents of human and bovine TB respectively. Human TB remains a significant source of morbidity and mortality with 9 million people developing TB and 1.5 million dying from this disease in 2013 (WHO, 2014). Similarly, bTB is increasing in incidence in regions of South West of England and Wales, and currently costs the UK economy up to £100 million per annum. An effective cattle vaccine is urgently required to improve control of bTB, alongside diagnostic tests which can differentiate infected from vaccinated animals (DIVA) (Krebs JR, 1997). However, currently within the UK there are no cattle vaccines licensed for use. Despite over a century of research, BCG remains the only vaccine available for use in humans against TB and is particularly effective when delivered to infants (Fine, 1995). Similarly, it is well established in the literature that experimental vaccination of neonatal calves with BCG provides significant protection against *M. bovis* infection (Buddle et al., 1995b, Hope et al., 2005, Hope et al., 2011), with protection induced by BCG vaccination lasting for at least 12 months (Thom et al., 2012). Nevertheless, BCG interferes with the tuberculin skin test and is therefore not licensed for use in the UK, and similar to humans (Fine, 1995), the efficacy of BCG is variable across studies in calves (Waters et al., 2012). Therefore improved vaccines are required and in order to design better vaccines, the immune response following BCG vaccination must be understood. Calves are immunocompetent at birth therefore respond well to neonatal vaccination and vaccination of neonates overcomes problems associated with exposure to environmental mycobacteria prior to vaccination. Furthermore, neonatal calves have an increased frequency (Kulberg et al., 2004, Graham et al., 2009) and activity (Elhmouzi-Younes et al., 2009) of NK cells which may explain the enhanced efficacy of BCG in neonatal calves.

Research within this thesis has focussed on the potential role of NK cells and NK cell interactions with DCs during BCG vaccination of neonatal calves which have not been previously investigated. Bovine NK cells are identified by their expression of NKp46 and the development of a mAb specific to this natural cytotoxicity receptor has

revolutionised bovine NK cell research (Storset et al., 2004). NK cells can be subdivided into CD2+ and CD2- subsets which differ in their localisation, phenotype and function (Boysen et al., 2006). In recent years, NK cells have been recognised as key players in both the innate and the adaptive immune response and accordingly, can interact with populations of accessory cells to drive adaptive immune responses. Protective immunity against *M. bovis* infection in cattle is hypothesised to be driven by Th1 immune responses (Buddle et al., 2005) which are driven by a cytokine milieu of IL-12 and IFN-γ. NK cells are major sources of IFN-γ and influence IL-12 production through interactions with APCs, therefore could be central to the induction of Th1 biased immunity.

NK cells are widely distributed in lymphoid and non-lymphoid compartments, allowing NK cells to respond quickly to infection and inflammation. In bovine PB, CD2+ NK cells constitute 70-80% of NK cells present whereas CD2- NK cells are the principal subset of NK cells found within bovine LNs. NK cells are hypothesised to enter LNs from the blood via HEVs or alternatively from tissues such as the skin via afferent lymphatic vessels (Martin-Fontecha et al., 2004, Bajenoff et al., 2006). Lund *et al* provided the first indication that bovine NK cells are present within skin-draining afferent lymphatic vessels by comparing samples of PB and AL from different animals (Lund et al., 2013). Those findings were extended in Chapter 3 of this thesis by comparing the frequency and phenotype of NK cells and NK cell subsets from PB, AL and LNs of the same animal. CD2- NK cells were the predominant NK cell subset present within AL and LNs, providing evidence that CD2- NK cells can migrate from the skin through afferent lymphatic vessels and into draining LNs in steady-state conditions. Furthermore, CD2- NK cells trafficking through AL were highly activated, as reflected by the expression of the activation marker CD25, and maintained this activated phenotype in the LNs. CD2+ NK cells were the main subset of NK cells resident within the skin which further strengthens the hypothesis that CD2- NK cells are the main migratory subset of NK cells in cattle. Furthermore, it was established in Chapter 3 that CD2- NK cells were the predominant subset of NK cells within the EL and can therefore egress from the LNs and return to the circulation where they were of a CD2+ $CD25^{lo}$ phenotype. The presence of NK cells within EL is a novel finding in

the bovine system, with NK cells having only been identified in human EL to date (Romagnani et al., 2007). Overall, findings presented in Chapter 3 provide the first indication that CD2- NK cells may represent a recirculating population of lymphocytes in cattle and as a result, may play a role in immune surveillance. Nevertheless, bilateral cannulation of the afferent and efferent lymphatic vessels in the same animal (as described by (Vrieling et al., 2012)) would establish if NK cells can re-circulate. NK cells would be isolated, fluorescently labelled and then injected subcutaneously back into the animal. The presence of labelled NK cells in both AL and EL would confirm that bovine NK cells can re-circulate and would reveal the kinetics of this process.

Further to determining the frequency and phenotype of PB, AL, LN and EL derived NK cells in steady-state conditions in Chapter 3, data presented in Chapter 4 assessed NK cell properties in the context of BCG vaccination. Studying NK cells draining the site of BCG vaccination will provide information about the activation of innate immune cells following vaccination. NK cells increased in frequency within the circulation following vaccination of neonatal calves with BCG and the receptor repertoire of NK cells was altered, indicating that NK cells in the blood undergo changes post-BCG vaccination. Conversely, NK cells within the LNs draining the site of BCG vaccination were not altered 24 or 48 hours post vaccination. As mentioned in Chapter 4, depletion of NK cells using a mAb specific for NKp46 prior to BCG vaccination and challenge with *M. bovis* would be the optimal method to define the functional role of NK cells during BCG-induced protection. Similar studies in a mouse model of *M. tb* infection showed that depletion of NK cells had no influence on the bacterial load within the lungs (Junqueira-Kipnis et al., 2003). The *in vivo* depletion technique has been successfully used to define the role of γδ T cells (Kennedy et al., 2002) and CD8+ T cells (Villarreal-Ramos et al., 2003) during *M. bovis* infection of cattle. It would also be of interest to assess the BCG-specific NK cell recall response by challenging NK cells from the studies described in Chapter 4 with PPD-b or BCG *in vitro*. Studies in cannulated calves provide a unique opportunity to study immune cells *ex vivo* which is particularly relevant for understanding immune responses following vaccination or infection. NK cell responses were studied in a small number

of afferent and efferent lymphatic cannulated calves and would need to be repeated to fully understand the mechanisms occurring at the site of BCG vaccination.

A key feature of NK cells is their ability to direct adaptive immune responses through interactions with populations of innate immune cells. As a result of *in vitro* co-culture with BCG-infected DCs, NK cells were highly activated and produced IFN-γ. Interestingly, CD2- NK cells were significantly more activated than CD2+ NK cells after co-culture with BCG-infected DCs and are also thought to preferentially produce IFN-γ (Boysen et al., 2006, Siddiqui and Hope, 2012). However, NK cell activation and production of IFN-γ was not observed when NK cells were cultured with BCG, uninfected DCs or cultured with DCs that had been exposed to Zylexis™ suggesting that in this system, NK cells require interactions with BCG-infected DCs to become activated. BCG-infected DCs expressed MHC class II, CD40 and CD80 and produced the Th1 polarising cytokine IL-12 and therefore propose that these molecules will play key roles in driving NK cell activation and effector function. Furthermore, this interaction may drive Th1 immune responses through the production of IL-12 and IFN-γ by BCG-infected DCs and NK cells respectively. To demonstrate a role for interactions between NK cells and DCs in driving Th1 immune responses in the context of BCG, autologous CD4+ or CD8+ T cells from BCG-vaccinated calves could be added into *in vitro* co-cultures between NK cells and DCs. Analysis of T-bet expression or production of IFN-γ by CD4+ T cells would decipher if NK cell-DC interactions were required for polarisation of Th1 responses in cattle. It has been demonstrated that NK cells drive Th1 polarisation of CD4+ T cells in mice (Martin-Fontecha et al., 2004) and human CD8+ T cell activation (Vankayalapati et al., 2004).

It is not known if the proposed *in vitro* interactions between NK cells and DCs infected with BCG occur *in vivo* in cattle. Innate immune cell interactions are expected to take place at sites of infection or inflammation and also within secondary lymphoid organs such as LNs (Cooper et al., 2004). Findings in Chapter 3 identified CD2- NK cells as the main NK cell subset which can migrate from skin through AL and into draining LNs therefore CD2- NK cells are located in AL and LNs where they may interact with DCs. CD2- NK cells preferentially express inflammatory and lymphoid homing

chemokine receptors including CXCR3, CD62L and CCR7 representing favoured migration of this subset of NK cells towards sites of vaccination or infection (Chapter 3 and (Siddiqui and Hope, 2012)). Data presented within Chapter 5 provided evidence that CD2- NK cells are the subset of NK cells which are activated following interactions with BCG-infected DCs. Furthermore, evidence presented in Chapter 4 supports a role for NK cells during BCG vaccination of neonatal calves. It may be that CD2- NK cells constitutively recirculate in steady-state conditions and upon BCG vaccination or *M. bovis* infection, may interact with DCs in draining LNs, and then exit the LNs to return to the site of vaccination or infection via the efferent lymphatic vessel. CD2- NK cells may also interact with BCG-infected DCs in skin draining LNs resulting in generation of Th1 biased CD4+ and CD8+ T cell responses. As a result of findings generated within this thesis, the proposed interaction between NK cells, DCs and BCG is illustrated in Figure 6.1.

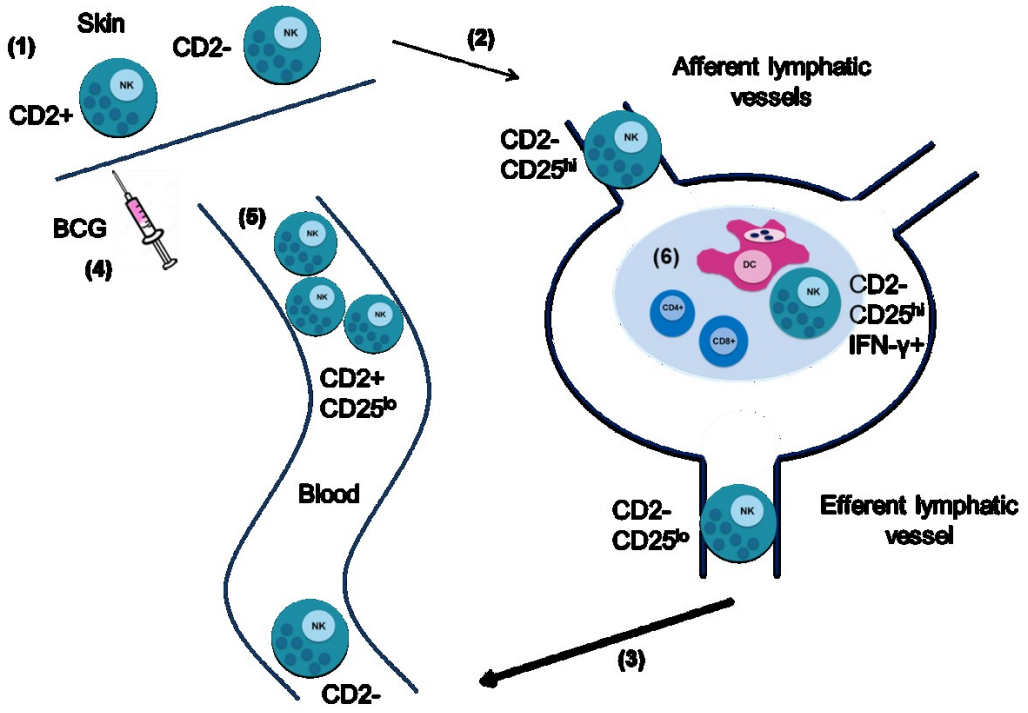


Figure 6.1. The proposed interaction between NK cells, DCs and BCG

CD2+ NK cells are the main subset of NK cells resident within bovine skin and may represent a non-migratory subset of NK cells (1). In steady-state conditions, CD2- NK cells can migrate from the skin through afferent lymphatic vessels and into LNs draining the skin due to their expression of LN homing molecules such as CD62L (2). CD2- NK cells are highly activated (as determined by expression of CD25) in AL and LNs compared with those found in the circulation. CD2- NK cells can then egress from the LNs to return to circulation via the efferent lymphatic vessel (3). Upon subcutaneous BCG vaccination (4), the frequency of NK cells within the circulation increased and changes to the receptor repertoire of NK cells were observed (5). Following vaccination, BCG-infected DCs may migrate to skin draining LNs where they preferentially interact with CD2- NK cells resulting in enhanced activation and effector function of NK cells and generation of a cytokine milieu conducive to the development of Th1 CD4+ and CD8+ T cells (6).

Interactions between NK cells and DCs have been the target for new therapies for cancer (Morandi et al., 2012, Shimizu and Fujii, 2009) and HIV (Altfeld et al., 2011). Similarly, the interaction between bovine CD2- NK cells and DCs may represent a target for future vaccination strategies to initiate Th1 polarised immune responses during BCG vaccination of neonatal calves. For example, NK cells are responsive to adjuvants (Magnusson et al., 2013) therefore adjuvants could be delivered alongside BCG to promote the co-localisation and interaction of CD2- NK cells and DCs which could subsequently drive NK cell activation and polarisation of Th1 immune responses.

To conclude, this thesis has provided evidence in support of the notion that CD2- NK cells preferentially migrate from the tissues into draining LNs via afferent lymphatic vessels and can then egress from the LNs to return to circulation via the efferent lymphatic vessels in steady-state conditions. In addition, it was demonstrated that NK cells may play a role in the immune response induced by BCG vaccination of neonatal calves, through increased frequency of NK cells and alterations to receptor repertoire. Finally, this thesis has shown that CD2- NK cells were preferentially activated following interactions with BCG-infected DCs and that together this interaction generated a cytokine milieu favourable to polarisation of Th1 immune responses which are important in the protective immune response against *M. bovis* infection of cattle. The findings produced in this thesis are not only relevant to improving vaccination strategies for enhanced control of bTB but may also be important for human vaccine design or delivery strategies for human TB as BCG vaccination of infants involves induction of NK cell responses and interactions between NK cells and APCs are important for *M. tb* specific immune responses.

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8.1 Media

Tissue Culture Medium

450ml RPMI 1640 with Glutamax + 25mM HEPES

10% Foetal Bovine Serum

2500µl Gentamicin

500µl 2-Mercaptoethanol

Middlebrook 7H9 medium

2.35g Middlebrook 7H9 broth base

450ml distilled water

0.5g Tween 80

1 vial Middlebrook ADC Supplement

Middlebrook 7H11 agar

21g Middlebrook 7H11 powder

900ml distilled water

100ml Middlebrook OADC enrichement

0.1M Ammonium Chloride Buffer

10mM $KHCO_3$

150mM NH_4Cl

0.1mM EDTA

PBS/1% BSA/0.1% sodium azide

500ml PBS

0.5g BSA

0.05g Sodium Azide

PBS/Tween 20

1000ml PBS

500µl Tween 20

PBS/0.5%BSA/2mM EDTA

896ml PBS

100ml 5% BSA

4ml 0.5M EDTA

20 x PMA/Ionomycin/Brefeldin A

50µl 100µg/ml PMA

100µl 1mg/ml Ionomycin

800µl 1.25mg/ml Brefeldin A

4.05ml PBS

FACSFlow/1%BSA

FACS Flow

1% BSA

8.2 Monoclonal antibodies

Antigen	Antibody	Isotype	Specificity	Supplier
NKp46	CD335-PE	IgG1	Mouse anti-bovine	AbD Serotec, UK
NKp46	EC1.1	IgG1	Mouse anti-ovine	Kindly provided by Tim Connelley
NKp48	AKS6	IgG2b	Mouse anti-bovine	Kindly provided by Anne Storset
CD2	CC42 - FITC	IgG1	Mouse anti-bovine	AbD Serotec, UK
CD2	ILA42	IgG2a	Mouse anti-bovine	IAH, Compton
CD3	MM1A	IgG1	Mouse anti-bovine	VMRD, Washington
CD25	CACT108A	IgG2a	Mouse anti-bovine	VMRD, Washington
CD62L	CC32-FITC	IgG1	Mouse anti-bovine	AbD Serotec, UK
CCR7 (CD197)	3D12-Alexa Fluor 647	IgG2a	Rat anti-human	BD Pharmingen
MHC class II DQ	CC158 - PE	IgG2a	Mouse anti-bovine	AbD Serotec, UK
CD40	ILA156 - FITC	IgG1	Mouse anti-bovine	AbD Serotec, UK
CD80	ILA159	IgG1	Mouse anti-bovine	IAH, Compton
CD86	ILA190	IgG1	Mouse anti-bovine	IAH, Compton
CD4	CC8	IgG2a	Mouse anti-bovine	IAH, Compton
CD8αα	CC63	IgG2a	Mouse anti-bovine	IAH, Compton
CD8αβ	CC58	IgG1	Mouse anti-bovine	IAH, Compton
WC1	CC15 - FITC	IgG2a	Mouse anti-bovine	AbD Serotec, UK
NKp30	F936 FH1	IgG2b	Mouse anti-bovine	IAH, Compton
IFN-γ	CC330-Alexa Fluor 647	IgG1	Mouse anti-bovine	AbD Serotec, UK
IFN-γ	CC330	IgG1	Mouse anti-bovine	AbD Serotec, UK
IFN-γ	CC302	IgG1	Mouse anti-bovine	AbD Serotec, UK
IL-12	CC301	IgG2a	Mouse anti-bovine	AbD Serotec, UK
IL-12	CC326	IgG2b	Mouse anti-bovine	AbD Serotec, UK

8.3 Fluorochromes

Specificity	Supplier
Goat anti-mouse IgG RPE	Southern Biotech
Goat anti-mouse IgG1 Alexa Fluor 647	Life Technologies
Goat anti-mouse IgG1 FITC	Bio-Rad
Goat anti-mouse IgG2a Alexa Fluor 647	Life Technologies
Goat anti-mouse IgG2b PE	Life Technologies

8.4 PCR primers

	Forward Primer	**Reverse Primer**
KLRC 1.2	TAAAAGTTCCATTTCAATGACT	CCAGGATCCCAGCAATGAA
KLRC 2.1	ACTCCAGGAAACAGCAAGTT	GTTATTCTGTTCCTGTATTAGAGC
KLRC 2.2	GGAGACAGCAAATGAGAGATTC	TCGGGAGCTTTGTTACCAG
GAPDH	GATGCTGGTGCTGAGTATGTAGTG	ATCCACAACAGACACGTTGGGAG

8.5 qPCR primers and mastermix compositions

	Forward Primer	**Reverse Primer**
NKp30	GTTGTTCATCTTTATCATCATCCG	ACTCCCTTGGCTGGCATTG
ATP5B	CCTTCTGCTGTGGGTTATCA	CAGGATCCGTCAAGTCATCA
EIF2B2	GAGCATATCCACTCCAACGA	CACTCTGCCACAATGACATG

	NKp30	***ATP5B***	***EIF2B2***
c DNA (µl)	1	1	1
Forward primer (µl)	0.5	2.1	2.1
Reverse primer (µl)	0.5	2.1	2.1
Mastermix (µl)	10	10	10
Water	8	4.8	4.8

8.6 ELISA reagents

	Capture monoclonal antibody	Detection monoclonal antibody	Cytokine standards
IFN-γ	Mouse anti-bovine CC330 (IgG1)	Mouse anti-bovine CC302 (IgG1)	COS cell derived recombinant bovine IFN-γ (IAH, Compton)
IL-12	Mouse anti-bovine CC301 (IgG2a)	Mouse anti-bovine CC326 (IgG2b)	COS cell derived recombinant bovine IL-12 (IAH, Compton)

8.7 Suppliers

AbD Serotec
Mouse serum
BD Biosciences
40μm cell strainers
BD LSRFortessa
FACS Permeabilising Solution 10X
FACSFlow
Middlebrook 7H11 agar
Middlebrook OADC enrichement
Biolegend
Zombie Aqua Fixable Viability Kit
Bioline
10x NH4 Reaction Buffer
1kb Hyperladder
50mM MgCl2
Agarose
BioTAQ DNA polymerase
d NTP mix
BioTek
Synergy HT Multi-Mode Microplate Reader
GE Healthcare UK Limiited
Streptavidin-HRP
GP Pharmaceuticals
Monoparin Heparin
Infusion Concepts
350ml blood bags
Invitrogen
RPMI 1640 with Glutamax and HEPES buffer 25mM
Superscript III Reverse Transcriptase system
SYBR Safe DNA gel stain
Life Technologies
DynaMag-15
Pan-mouse IgG Dynabeads
MicroAmp Fast Optical 96-Well Reaction Plate q PCR plates
Sytox Blue Nucleic Acid Stain
Luminaris Colour HiGreen Low ROX qPCR master mix
Miltenyi Biotech
Human CD14 MicroBeads
MACS LS columns
MACS Multi stand
Midi MACS magnet

Movianto
BCG Danish SSI
Sauton Medium
Prionics Ag
Avian PPD
Bovine PPD
Promega
dNTPs
Taq polymerase
10x PCR reaction buffer
$MgCl_2$
Qiagen
Qiashredders
RNeasy mini kit
Buffer RLT
RNase-Free Dnase Set
Sarstedt
500ml blood bags
Schering-Plough
Sodium benzylepenicillin
Sigma Aldrich
2-Mercatoethanol
7H9 Middlebrook Broth Base
Bovine Serum Albumin
Brefeldin A
Carbonate-bicarbonate buffer capsules
Cell Dissociation Solution Non-Enymatic 1x
Collagenase
Dispase
Dimethyl sulfoxide (DMSO)
Ethylenediaminetetraacetic acid (EDTA)
FITC isomer
Gentamicin
Histopaque 1083
Ionomycin
Paraformaldehyde
Phorbol 12-myristate 13-acetate (PMA)
Sodium Azide
Sodium Casein
3,3',5,5'-Tetramethylbenzidine (TMB) substrate
Tween-20
TCS Biosciences Ltd
Foetal Bovine Serum

Terumo
50ml syringes
21G needles
Thermo Fisher Scientific
0.2ml thin walled 8 tube strips
96 well round bottom plates
Disposable scalpels
Heparin Sulphate
Nunclon Delta Surface 12 well plate
Nunclon Delta Surface 24 well plate
Nunclon Delta Surface 6 well plate
Superscript III Reverse Transcriptase system
T75 flasks
TMB substrate
Trypan Blue
VWR International
NUNC Maxisorp ELISA plate